Implication of macrophage inflammatory protein-1α in the inhibition of human haematopoietic progenitor growth by dengue virus

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Dengue viruses occur as four distinct serotypes: dengue 1, 2, 3 and 4 (DEN-1, DEN-2, DEN-3 and DEN-4) which can induce a large spectrum of clinical manifestations ranging from a mild disease (dengue fever) to life-threatening forms (dengue shock syndrome; DHF/DSS). Whatever the clinical symptoms, dengue infection is frequently associated with haematological disorders such as leukopenia and thrombocytopenia, and the bone marrow is markedly hypocellular during the febrile phase of the disease (Bierman & Nelson, 1965; Halstead, 1982; Nelson & Bierman, 1964), suggesting an association of dengue with bone marrow failure. We previously demonstrated (Murgue et al., 1997) an in vitro inhibition of haematopoietic progenitor growth induced by several clinical isolates of DEN-3 and observed differences in the level of the inhibitory effect between the isolates tested.

The aim of this study was to investigate the mechanisms of the haematopoietic progenitor growth inhibition observed after in vitro dengue infection of cord blood mononuclear cells (CBMNC). For this purpose, the effects were compared of an isolate of DEN-3 (29-56DSS) obtained from a child with a primary fatal grade IV DSS, previously shown to be a very potent inhibitor of progenitor growth, with those induced by the prototype strain of DEN-3 (H-87), which had no such effect (Murgue et al., 1997). Both viruses were low-passaged on mosquito cells and titred on C6/36 cells (Chungue et al., 1993). They were used at a low m.o.i. of 10⁻² TCID₅₀ per cell, at which the 29-56DSS isolate induced an inhibition of 90%. Culture fluid from dengue-negative serum or culture medium alone were used as negative controls.

Inoculation and liquid culture were performed as described (Murgue et al., 1997). Briefly, fresh, non-adherent CBMNC were incubated for 2 h at 37 °C with either 29-56DSS isolate or H-87. Thereafter, cells were harvested, washed and seeded in liquid culture at 37 °C for up to 8 days with interleukin-3 (IL-3), IL-6 and stem cell factor from the Media Preparation Service, Terry Fox Laboratory, Vancouver, Canada. At days 1, 3, 6 and 8, cells were harvested, washed, counted and seeded (2–3 x 10⁴ cells) in methylcellulose for clonogenic assays of haematopoietic progenitors as described by Guigon et al. (1990), in the presence of agar–leukocyte-conditioned medium (y/v) and 3 U/ml erythropoietin (Terry Fox Laboratory). The numbers of colony-forming unit granulocyte macrophages (CFU-GM) and burst-forming unit erythroids (BFU-E) were scored under the microscope between 14 and 18 days of culture. Because of the low number of BFU-E generated by control cultures at D8, the results were expressed as the total number of colonies (CFU-GM + BFU-E). Statistical analysis was carried out using analysis of variance and the Newman–Keul test.

The number of viable cells recovered at days 1, 3, 6 and 8 of liquid culture was not different in H-87 infected cultures as compared with control experiments. By contrast, in day 6 and

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inhibitor of progenitor growth as early as the sixth day of liquid culture.

We then looked for the mechanism of this inhibition. It has previously been shown that at day 8 of liquid culture, virus replication was not different in 29-56DSS compared with H-87 infected cultures (Murgue et al., 1997). However, these results did not mean that the replication of 29-56DSS isolate before the eighth day of culture was similar to that of H-87. Therefore, virus replication was analysed for both viruses throughout the liquid culture. Fifty µl cell-free supernatant and 5 × 10⁴ cells from days 1, 3, 6 and 8 cultures infected by either virus were inoculated in duplicate onto C6/36 cells. Because of the low numbers of virus particles in cells and supernatants, virus titres were measured by counting fluorescent foci (Igarashi & Mantani, 1974). Viral antigens were inconstantly detected in cells and supernatants, whatever the virus tested and the day of culture. Furthermore, when virus particles were detected, the titre was very low (< 50 fluorescent foci/ml and < 5 fluorescent foci/10⁶ cells). In addition, RT–PCR was performed on cells and supernatants, using the method of Chomczynski & Sacchi (1987) for RNA extraction, and a semi-nested PCR as described by Lanciotti et al. (1992). The results (Fig. 1b) showed that dengue virus RNA was almost constantly detected whatever the virus tested and the day of culture. There was no obvious difference in the level of dengue virus RNA between the two viruses. Therefore it did not appear that the progenitor growth inhibition induced by 29-56DSS isolate was related to the level of virus replication.

When CD34⁺ cells, purified from CBMNC, were infected with 29-56DSS isolate at an m.o.i. of 10⁻² and then seeded in liquid culture for up to 8 days, no inhibition of progenitor growth could be detected, either 2 h after infection or after 4, 6 and 8 days of culture (data not shown). Similar results were obtained when CD34⁺ cells were infected at a higher m.o.i. of 1. These results demonstrated that dengue virus did not affect the growth of CD34⁺ cells, and suggested that the effect of dengue virus on haematopoiesis is probably not direct.

The inhibitory effect of dengue virus on progenitor growth may be related to the induction of factor(s) released by the infected CBMNC. To test this hypothesis, supernatants from days 3, 6 and 8 infected and non-infected liquid cultures were recovered and centrifuged to eliminate cells. Aliquots of 400 µl were inactivated at 56 °C for 30 min. The absence of infectious particles was verified by inoculation on C6/36 cells. These heat-inactivated supernatants were added to methylcellulose cultures of normal fresh CBMNC at a final concentration of 10% (v/v). The numbers of BFU-E and CFU-GM colonies were evaluated after 14 days of culture, and were not different when experiments were conducted in the presence of heat-inactivated supernatants generated by either H-87 or control cultures (Fig. 2). By contrast, with day 3 heat-inactivated supernatants of 29-56DSS infected cultures, a slight but significant inhibition (18 ± 3%) of CFU-GM was observed (P < 0.01) compared with control cultures. Moreover, day 6...
inactive viral particles cannot be completely excluded. The inhibition was still observed with day 8 supernatants (\(P < 0.05\)) in supernatants of 29-56DSS cultures compared with those observed in control cultures (\(P < 0.001\) and \(P = 0.0003\), respectively). The inhibition was still observed with day 8 supernatants (\(P < 0.05\)) for both BFU-E and CFU-GM. These results indicated the presence of factor(s) with suppressive activities on haematopoietic progenitor growth in supernatants of 29-56DSS infected cultures. However, a potential role of inactive viral particles cannot be completely excluded.

Levels of tumour necrosis factor-\(z\) (TNF-\(z\)), transforming growth factor-\(\beta\) (TGF-\(\beta\)) and macrophage inflammatory protein-1\(z\) (MIP-1\(z\)), known for their inhibitory effect on haematopoiesis (Bonnet et al., 1995), were measured by ELISA. Assays (R&D Systems) were performed in days 1, 3, 6 and 8 cell-free supernatants of cultures infected by either virus, or non-infected. TNF-\(z\), known for its pleiotropic effects, was found at a higher level in sera of patients with DHF/DSS compared with patients with mild dengue fever (Hober et al., 1993; Yadav et al., 1991). Furthermore, Anderson et al. (1997) demonstrated that dengue virus could modulate endothelial cell function by an indirect route, in which a key intermediary would be TNF-\(z\) released from virus infected monocytes. In these experiments \((n = 5)\), TNF-\(z\) levels were not significantly different \((P > 0.05)\) in supernatants of 29-56DSS infected cultures as compared with those obtained in H-87 infected or non-infected cultures (range 70–240 pg/ml).

Zauli et al. (1996) demonstrated that the suppressive effect induced by human immunodeficiency virus (HIV) on CD34+ haematopoietic progenitor cells was mediated by an up-regulation of TGF-\(\beta\) endogenously produced by CD34+ cells. In our experiments \((n = 3)\), TGF-\(\beta\) levels were not different \((P > 0.05)\) in supernatants of 29-56DSS cultures compared with H-87 infected or non-infected cultures (range 446–1131 pg/ml).

MIP-1\(z\) is an 8 kDa C–C chemokine produced by a variety of cell types (Graham et al., 1990), which inhibits the proliferation of primitive progenitors (Bonnet et al., 1995). An increase in MIP-1\(z\) levels has been recently demonstrated in HIV infected blood-derived macrophages, suggesting a role of this protein in impaired haematopoiesis or as a CD4+ and CD8+ lymphocyte chemoattractant (Canque et al., 1996).

The results of six separate experiments are shown in Fig. 3(a). Similar patterns of MIP-1\(z\) production in both H-87 infected and non-infected cultures were found throughout the liquid culture. By contrast, the levels of MIP-1\(z\) in 29-56DSS infected cultures at day 6 were significantly higher compared with those observed in control cultures (\(P < 0.001\)) and remained generally high at day 8, despite great variations between experiments (\(P < 0.01\); range 55–3873 pg/ml for 29-56DSS, versus 23–195 pg/ml for control experiments). MIP-1\(z\) levels were not different in supernatants before and after heat-inactivation (data not shown). The increase in the level of MIP-1\(z\) was not associated with any marked change in mRNA expression in cells infected by 29-56DSS isolate compared with H-87 infected and non-infected cultures. Fig. 3(b) depicts representative results of the amplification of an MIP-1\(z\) RNA fragment of 174 bp performed by using specific primers for the first round RT–PCR (Canque et al., 1996), and followed by a semi-nested PCR using an internal genomic-sense primer (5’ GGACACCTGGAGCTGAGGCC). To normalize the quantity of RNA, PCR was performed in parallel with \(\beta\)-actin primers.

Thus, in our experiments, the inhibitory effect induced by day 6 and day 8 supernatants of 29-56DSS infected cultures paralleled that obtained with higher levels of MIP-1\(z\), as compared with H-87 infected or control cultures. The fact that MIP-1\(z\) is an inhibitor for primitive progenitors (Bonnet et al., 1995) may account for the delayed appearance of inhibition in 29-56DSS infected cultures. Although protein levels in the experiment shown in Fig. 3(c) were significantly increased in day 6 29-56DSS supernatant (but not in day 8 in this particular experiment) as compared with the control, no corresponding difference in mRNA signals was noted.

![Fig. 2](image-url)
The results of six separate experiments showed that anti-MIP-1α induced a significant increase \((P < 0.05)\) in the number of viable cells in 29-56DSS infected cultures relative to non-supplemented cultures. No significant increase was observed in control cultures.

The total number of colonies was not significantly increased in day 8 control cultures supplemented with anti-MIP-1α antibody as compared with non-supplemented cultures. By contrast, in 29-56DSS infected cultures, there was a significant increase in the number of colonies \((3.83\text{-fold}; \text{range} 1.43–10.42)\) after addition of anti-MIP-1α antibody as compared with non-supplemented cultures \((P < 0.025)\); furthermore, the size of BFU-E and CFU-GM colonies became comparable to those observed in control cultures. However, despite the significant increase in the number of colonies induced by anti-MIP-1α antibody in 29-56DSS cultures, the inhibition remained high \((80\%)\). This could be due either to the dose or to the schedule of application of the antibody, and/or to the participation of other, as yet unidentified factors. Taken together, these data suggest that the higher levels of MIP-1α might account for some aspects of impaired haematopoiesis during dengue infection. However, the suggestion cannot be excluded that the inhibition of progenitor growth observed in 29-56DSS infected cultures may also be related to an impairment of the production of stimulating cytokines; this remains to be determined.

To our knowledge, this is the first report demonstrating that the potential mechanism of the \textit{in vitro} haematopoietic suppression associated with dengue infection could be due to an indirect effect of dengue virus on haematopoiesis, through the release of haematosuppressive factors such as MIP-1α.

The implication of MIP-1α was confirmed by blocking experiments using a goat anti-human MIP-1α antibody (R&D Systems) added at day 0, in 29-56DSS infected or non-infected cultures, at a final concentration of 100 µg/ml. The number of viable cells and of clonogenic progenitors was determined at day 8 of liquid culture.

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References


MIP-1α and dengue induced haematosuppression


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