Dengue Virus Infection of Mast Cells Triggers Endothelial Cell Activation

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Vascular perturbation is a hallmark of severe forms of dengue disease. We show here that antibody-enhanced dengue virus infection of primary human cord blood-derived mast cells (CBMCs) and the human mast cell-like line HMC-1 results in the release of factor(s) which activate human endothelial cells, as evidenced by increased expression of the adhesion molecules ICAM-1 and VCAM-1. Endothelial cell activation was prevented by pretreatment of mast cell-derived supernatants with a tumor necrosis factor (TNF)-specific blocking antibody, thus identifying TNF as the endothelial cell-activating factor. Our findings suggest that mast cells may represent an important source of TNF, promoting vascular endothelial perturbation following antibody-enhanced dengue virus infection.

We have previously reported that human mast cells are permissive to antibody-enhanced dengue virus infection, occurring in an FcyRII-dependent manner (6, 23). Infection of mast cell lines KU812 and HMC-1 results in infectious virus production and the induction of significant levels of cytokines, such as interleukin-1β (IL-1β) and IL-6, as well as chemokines CCL3, CCL4, and CCL5, which can recruit additional immune effector cells (22).

In this study, we determined whether antibody-enhanced, dengue virus-infected human mast cells produced factors that could activate human endothelial cells. Cord blood-derived mast cells (CBMCs) were prepared as previously described (6). Briefly, cells were cultured at an initial concentration of 0.6 × 10^6/ml in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), 100 U of penicillin per ml, 100 µg of streptomycin per ml, 20% CCL-204 cell supernatant as a source of IL-6, 10^−7 M prostaglandin E2 (Sigma), and 50 to 100 ng of stem cell factor (SCF; Peprotech, Rocky Hill, NJ)/ml for 5 to 10 weeks, with purity assessed by toluidine blue (pH 1.0) staining of cytocentrifuge preparations and examination of cells for the presence of multiple metachromatic granules and appropriate nuclear morphology. These cells are predominantly positive by flow cytometry for CD117 (c-kit) and CD13 but not CD14. Only mast cell preparations that were ≥95% pure, with a mean purity of 97%, were used for this study. CBMCs and the human mast cell-like line HMC-1 (7) were mock treated using conditioned medium from uninfected Vero cells or exposed (multiplicity of infection [MOI] of 1 to 3) to dengue virus alone or dengue virus or UV-inactivated dengue virus with a 1:10,000 dilution of pooled convalescent, dengue-immune patient sera (6). Supernatants were collected at 12, 24, and 48 h postinfection.

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in gelatin-coated flasks as described previously (1). Briefly, HUVECs were isolated from umbilical cords by collagenase (Cooper Biomedical, Mississauga, Ontario, Canada) treatment and grown in RPMI 1640 medium containing 2 mM l-glutamine, 50 µM 2-mercaptoethanol, 1...
mM sodium pyruvate, penicillin G, and streptomycin (Gibco BRL, Burlington, Ontario, Canada), supplemented with 20% FCS, endothelial cell growth supplement (25 µg/ml; Collaborative Research, Lexington, MA), and heparin (45 µg/ml; Sigma). Cells from the first to third passages were harvested with trypsin-EDTA, seeded into 96-well flat-bottom tissue culture plates in growth medium, and used as soon as confluent. Mast cell supernatants were added to HUVEC monolayers at a 1:3 dilution and supplemented with 10% FCS and 10% autologous human serum (Fig. 1). Following 18 h of incubation, the expression of adhesion molecules on viable HUVECs was determined by indirect enzyme-linked immunosorbent assay (ELISA) as previously described (1, 36). Briefly, HUVEC monolayers that had been exposed to mast cell supernatants were washed and incubated (60 min at 37°C) in RPMI 1640–10% FCS–0.1% sodium azide supplemented with 2 µg/ml anti-ICAM-1 or anti-VCAM-1 monoclonal antibody (R&D Systems). This was followed by washing and the addition of goat anti-mouse IgG-horseradish peroxidase conjugate (Can-Bio-science, Mississauga, Ontario, Canada) for 60 min at 37°C. After washing and the addition of orthophenylenediamine substrate (Sigma), color development was stopped with 4 N sulfuric acid, and absorbances at 490 nm were determined with an automatic ELISA reader.

For HUVECs exposed to HMC-1 supernatants, upregulation of ICAM-1 and VCAM-1 was greatest with exposure to 24-h supernatants from antibody-enhanced, dengue virus-infected cells (Fig. 1A and B), whereas upregulation of ICAM-1 and VCAM-1 on HUVECs exposed to CBMC supernatants was greatest with 12-h supernatants from antibody-enhanced, dengue virus-infected cells (Fig. 1C and D). Importantly, supernatants from mast cells stimulated with dengue virus alone or with UV-inactivated dengue virus plus antibody did not result in an increase of ICAM-1 or VCAM-1 expression over basal expression (mock infection). These findings indicate that antibody-enhanced dengue virus infection of human mast cells promotes the release of mediator(s) which can alter the activation status of endothelium.

In order to identify the HUVEC-activating factor, mast cell supernatants were preincubated for 30 min at 4°C with 10 µg/ml of blocking antibodies for likely candidates, including IL-1α, IL-1β, and tumor necrosis factor (TNF; previ-
ously known as TNF-α (Fig. 2A). Initially, we determined that TNF blockade prevented the upregulation of ICAM-1, while blockade of IL-1α or IL-1β had no effect (Fig. 2A). Subsequently, further experiments involving blockade of TNF showed significant prevention of upregulation of both ICAM-1 and VCAM-1 on HUVECs exposed to supernatants from antibody-enhanced dengue virus-infected CBMCs and HMC-1 cells (Fig. 2B). We have since determined that both CBMCs and HMC-1 cells do not produce any detectable IL-1α (<3.5 pg/ml) following antibody-enhanced dengue virus infection. Additionally, while HMC-1 cells do constitutively produce very low levels of IL-1β (20 pg/ml), this is unchanged following antibody-enhanced dengue virus infection. CBMCs do not produce any detectable IL-1β (<3.9 pg/ml) following antibody-enhanced dengue virus infection (data not shown). Previous reports have indicated that (20 ng/ml) IL-1β induces HUVEC upregulation (31). Importantly, these reported levels are at least 1,000 times higher than those produced by HMC-1 cells and CBMCs. Therefore, we conclude that both IL-α and IL-1β are not factors influencing HUVEC activation in our system. Collectively, these data reinforce the identification of TNF as the HUVEC-activating factor produced by antibody-enhanced, dengue virus-infected human mast cells.

Supernatants collected at 12, 24, and 48 h from antibody-enhanced, dengue virus-infected mast cells were analyzed by using sandwich ELISA to determine the concentrations of TNF released (Fig. 3A and B). Significant increases in TNF release by HMC-1 cells (Fig. 3A) and CBMCs (Fig. 3B) compared to the results for mock treatment occurred only in response to antibody-enhanced dengue virus infection. Neither inoculation with dengue virus alone nor UV-inactivated dengue virus with antibody triggered TNF release. This correlated with the endothelial activation, which only occurred in response to supernatants from antibody-enhanced, dengue virus-infected mast cells. These results also indicate that dengue
It is important to note that another mast cell line, KU812, does not produce appreciable amounts of TNF in response to antibody-enhanced dengue virus infection (23). This appears to be an anomaly particular to KU812 cells, as CBMCs and HMC-1 cells respond to antibody-enhanced dengue virus infection with TNF production (Fig. 3).

We have previously shown that infection of mast cells by dengue virus is dependent upon antibody-virus complexes binding through FcγRII (6). We show here, further, that in the absence of dengue virus antibody, even high-MOI dengue virus inoculation is ineffective in eliciting infection or TNF production (23).

Therefore, antibody-enhanced dengue virus infection of mast cells does not result in enhanced TNF production; more accurately, binding of virus-antibody complexes represents the primary mechanism of human mast cell infection, leading to the subsequent production of TNF in addition to other inflammatory mediators (22). Productive dengue virus infection of human mast cells does not occur in the absence of anti-dengue virus antibodies.

Previous reports have linked dengue-induced TNF to changes in endothelial permeability, leading to leakage (10, 13). We therefore investigated whether TNF-containing supernatants from dengue virus-infected mast cells could affect the permeability of HUVECs. Confluent HUVEC monolayers on 0.4-μm transwells were stimulated with supernatants and then assayed for leakage at 20, 40, and 80 min following the addition of 125I-albumin. In our hands, we did not detect any significant changes in endothelial permeability induced by supernatants from CBMCs or HMC-1 cells or by recombinant TNF stimulation (3 ng/ml) (Fig. 4). Thrombin as a positive control (10 U/ml) was effective at promoting leakage, as determined by the increased movement of 125I-albumin across the HUVEC monolayer. Therefore, despite the activation of adhesion molecules ICAM-1 and VCAM-1 on endothelial cells, permeability was unchanged following exposure to TNF-containing supernatants from dengue virus-infected mast cells. Previous reports of TNF-induced permeability of HUVECs required very high concentrations of TNF (10 to 1,000 ng/ml), in some instances, in combination with ongoing dengue virus infection (13, 14, 30). However, the physiological relevance of such effects is uncertain since the average TNF levels detected in DHF patient sera are 20 to 40 pg/ml (3, 11, 24, 40).

These results verify the integrity of the endothelial cells used in these studies. Importantly, the results suggest a role for dengue virus-infected mast cells in endothelial cell activation but not permeability, for which other cell-derived factors are likely required (4, 10, 13).

This is the first report of antibody-enhanced dengue virus infection of human mast cells promoting the production and release of TNF, with effects on human endothelial cells. TNF is a potent endothelial cell-activating factor with recognized importance in the pathogenesis of severe dengue disease (1,

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**TABLE 1. Dengue virus infection of human mast cells is antibody dependent, leading to TNF production**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of E protein-positive cells</th>
<th>Amt of TNF (pg/ml) produced by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMC-1</td>
<td>CBMCs</td>
</tr>
<tr>
<td>Mock</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>High-MOI DV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV high-MOI DV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DV + Ab</td>
<td>18.18 ± 7.83</td>
<td>2.79 ± 1.38</td>
</tr>
<tr>
<td>UV DV + Ab</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are the average results of three separate experiments ± standard deviations.
High levels of TNF in DHF patients are suspected of causing activation or injury of endothelial cells (20, 38, 39, 49). Clinical studies of dengue virus-infected patients have repeatedly found high levels of TNF in serum, with increased levels strongly correlated with the severity of disease (16, 20, 27). Blockade of TNF has been reported to decrease morbidity in a mouse model of dengue virus infection (2).

TNF derived from dengue virus-infected monocytes and activated T cells has previously been proposed as the major mediator responsible for the dysregulation of endothelial homeostasis in vitro and hypothesized to be a prominent mediator leading to plasma leakage in vivo (1, 17, 26). The results from the present study suggest that dengue virus-infected mast cells may be an additional significant source of TNF. Importantly, TNF is a known inducer of subsequent inflammatory cytokines (IL-6) and can enhance the production of lipid mediators (platelet-activating factor, leukotrienes, prostaglandins, and IL-6) and can enhance the production of lipid mediators (28). Additionally, coculture of peripheral blood mononuclear cells promotes changes in dengue virus-infected HUVECs, including decreased vascular endothelial cadherin expression and increased permeability (14). Finally, TNF has been shown to enhance dengue-induced NO and reactive oxygen species effects on endothelial cell damage and hemorrhage in a mouse model of dengue virus infection (50).

The results of the present study add mast cells and mast cell-derived TNF to the select group of cell mediators of dengue-induced endothelial cell perturbation.

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REFERENCES


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