Activation of Endothelial Cells via Antibody-Enhanced Dengue Virus Infection of Peripheral Blood Monocytes

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Although endothelial cells have been speculated to be a target in the pathogenesis of dengue hemorrhagic fever (DHF), there has been little evidence linking dengue virus infection to any alteration in endothelial cell function. In this study, we show that human umbilical vein endothelial cells become activated when exposed to culture fluids from dengue virus-infected peripheral blood monocytes. Maximum activation was achieved with culture fluids from monocytes in which virus infection was enhanced by the addition of dengue virus-immune serum, thus correlating with epidemiological evidence that prior immunity to dengue virus is a major risk factor for DHF. Activation was strongest for endothelial cell expression of VCAM-1 and ICAM-1. In contrast, activation of endothelial cell E-selectin expression appeared to be more transient, as indicated by its detection at 3 h, but not at 16 h, of treatment. Treatment of monocyte culture fluids with anti-tumor necrosis factor alpha (TNF-α) antibody largely abolished the activation effect (as measured by endothelial cell expression of ICAM-1), whereas treatment with IL-1β receptor antagonist had a much smaller inhibitory effect on activation. Endothelial cells inoculated directly with dengue virus or with virus-antibody combinations were poorly infectable (compared to Vero cells or peripheral blood monocytes), and virus-inoculated endothelial cells showed no increased expression of VCAM-1, ICAM-1, or E-selectin. Taken together, the results strongly indicate that dengue virus can modulate endothelial cell function by an indirect route, in which a key intermediary is TNF-α released from virus-infected monocytes.

**MATERIALS AND METHODS**

Reagents and MAbs. Recombinant human tumor necrosis factor alpha (TNF-α) and human interleukin-1β (IL-1β) were gifts from Genentech, Inc. (South San Francisco, Calif.), and Immunex (Seattle, Wash.), respectively. Neutralizing monoclonal antibody (MAb) to human TNF-α was a gift from Genentech, and IL-1 receptor antagonist (IL-1RA) was a gift from Synergen (Boulder, Colo.). Mouse MAbs to adhesion molecules were obtained as follows: RR1/1 (immunoglobulin G1 [IgG1] anti-ICAM-1) from T. A. Springer (Boston, Mass.); IL-1 receptor antagonist (IL-1RA) was a gift from Synergen (Boulder, Colo.), Mouse MAbs to adhesion molecules were obtained as follows: RR1/1 (immunoglobulin G1 [IgG1] anti-ICAM-1) from T. A. Springer (Boston, Mass.); and 4B9 (IgG1 anti-VCAM-1) and DA5 (IgG1 anti-E-selectin) from R. Lobb (Biogen, Cambridge, Mass.). An isotype-matched, irrelevant control MAb (3H11B9, anti-pertussis toxin) was a gift from S. Halperin, Department of Pediatrics, Dalhousie University, Halifax, Nova Scotia, Canada.
Cells, virus, and immune serum. Dengue virus type 2 strain 16681 (20) was grown in monolayer cultures of Vero cells, maintained in Eagle’s modified minimal essential medium supplemented with 5% fetal calf serum (FCS) as previously described (21). Virus titer was 2×10^6/ml; virus inoculations of Vero cell monolayers, and endothelial cells were carried out at a multiplicity of infection (MOI) of 0.1. For some experiments, virus was incubated by exposure to short-wave UV from a germicidal lamp for 10 min; inactivation of virus infectivity was verified by plaque assay on LLC-MK2 cells. Dengue virus-immune sera, obtained from dengue-convalescent patients, were kindly provided by B. Innis and A. Nisalak, AFRIMS, Bangkok, Thailand. The collection of these sera has been described previously (21). Briefly, the original collection contained both acute- and convalescent-phase sera obtained from patients who were initially viremic with dengue virus type 2. For the present study, we chose four convalescent-phase sera which showed at least an eightfold increase in anti-dengue virus IgG over the acute-phase serum samples, as determined by isotype capture enzyme immunoassay (25). Fifty percent plaque reduction neutralization titers against dengue virus type 2 on LLC-MK2 cells for the four sera ranged between 1:80 and 1:640 (i.e., serum 1036, 1:80; serum 6449, 1:80; serum 7705, 1:160; and serum 7873, 1:640). Dengue virus type 2 enhancing titers for the four sera, assessed on human peripheral blood monocytes (18), ranged between 1:640 and 1:12,240. Additional control sera were taken from healthy dengue virus-immune donors in the Department of Microbiology & Immunology, Dalhousie University.

Preparation of peripheral blood monocytes and infection with dengue virus. Blood was obtained from mononuclear cell fractions from healthy, dengue virus-immune, adult volunteers (Department of Microbiology & Immunology, Dalhousie University) by venapuncture and collection into heparinized tubes. Donors were not screened for immunity against other flaviviruses, although donors were indicated no prior exposure to flaviviruses. Collection of blood was processed immediately by standard methods for cell preparation and fractionation. Briefly, mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 (density of 1.077 g/ml; Sigma, St. Louis, Mo.). Hepatinized blood was mixed 1:1 with RPMI 1640 without added serum, layered over the Histopaque-1077, and centrifuged at room temperature for 30 min at 400×g. The cell layer at the interface, consisting mainly of mononuclear cells, was collected and washed twice in RPMI 1640 without serum by centrifugation at 400×g for 10 min. The mononuclear cells obtained were resuspended to 2×10^6 cells/ml in RPMI 1640 supplemented with 25 mM HEPES (BDH Laboratory Supplies, Edmonton, Alberta, Canada), 2 mM additional l-glutamine (Gibco BRL, Burlington, Ontario, Canada), penicillin (100 U/ml; Gibco BRL), streptomycin (100 μg/ml; Gibco BRL), and 5% FCS. Cells were incubated in 48-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J.) for 12 h at 37°C, and the nonadherent cells were removed by being washed five times. The remaining adherent cells, typically >90% monocytes, were adsorbed (90 min at 4°C) with aliquots of dengue virus, UV-inactivated dengue virus, dengue virus-immune serum, or combinations of virus and immune serum (premixed for 60 min at 4°C). Dengue virus was used at an MOI of 0.1, consistent with previous procedures to achieve optimum antibody-dependent enhancement (5, 18). As a positive control, lipopolysaccharide (LPS) at a concentration of 1 ng/ml was added to two series of wells, one kept at 4°C during the adsorption step and the other kept at 37°C for the same time period. The cells were then washed three times and incubated in 400 μl of medium at 37°C. Aliquots of 150 μl were removed at various times postinfection and stored at −70°C until used for addition to endothelial cell cultures.

Endothelial cell culture. Human umbilical vein endothelial cells were isolated and cultured in gelatin-coated flasks as described by Jaffe et al. (27), with modifications reported previously (26). Briefly, endothelial cells were obtained from umbilical cords after treatment with 0.5 mg of collagenase (Cooper Bio-medical, Mississauga, Ontario, Canada) per ml in 0.1 M phosphate-buffered saline (pH 7.4) and grown in RPMI 1640 containing 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, penicillin G, and streptomycin (Gibco BRL) and supplemented with 20% FCS, 250 endothelial cell growth supplement (25 μg/ml; Collaborative Research, Lexington, Mass.), and heparin (45 μg/ml; Sigma). Cells from the first to third passages were harvested by using trypsin-EDTA and seeded into 96-well flat-bottom tissue culture plates in growth medium and used as soon as confluent.

Radiolabeling of peripheral blood monocytes, endothelial cells, and Vero cells with [35S]methionine-[35S]cysteine. Cultures of monocytes, endothelial cells, and Vero cells were labeled with [35S]methionine-[35S]cysteine (Translabel; ICN, Montreal, Quebec, Canada) at a concentration of 200 μCi/ml in methionine- and cysteine-free medium from 36 to 48 h postinfection. Culture fluids were harvested at 48 h postinfection, clarified by centrifugation, and immunoprecipitated with human dengue virus-immune serum and protein A-bearing, formalin-fixed Staphylococcus aureus as previously described (21). Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radiolabeled proteins were visualized by fluorography (21).

Antibody-dependent enhancement of dengue virus infection of monocytes. Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, Calif.) were used, since MOIs of 0.1 or less have been shown previously to be optimal for antibody-dependent enhancement of infection by dengue virus (5, 18). For comparison, inoculation of Vero cells (which do not have Fc receptors) with the same virus and virus-antibody mixtures showed no analogous depen-
dence on antibody, as indicated by similar yields of radiolabeled viral proteins produced in all samples tested (Fig. 1).

Using the conditions for monocyte culture and infection established above, we examined the hypothesis that infected monocytes might secrete a factor(s) which affects endothelial cell properties. Monocyte culture fluids harvested at 36 h were added to endothelial cell cultures and incubated for 16 h in order to examine upregulation of adhesion molecules VCAM-1, ICAM-1, and E-selectin. Parallel wells of endothelial cells were treated with IL-1β or TNF-α as positive controls. As shown in Fig. 2, no induction of adhesion molecule expression was observed by culture supernatants from monocytes which had been inoculated with virus alone or with virus-immune antibody alone. However, VCAM-1 and to a lesser extent ICAM-1 were induced by culture fluids from monocytes inoculated with virus-antibody (1:640 and 1:2,560) combinations, which were the same combinations which resulted in maximal virus infection (Fig. 1). In contrast to VCAM-1 and ICAM-1, there was no detectable elevation in the expression of E-selectin, as assayed after 16 h of treatment.

Because the expression of E-selectin can be activated rapidly and in a short-lived manner by certain stimuli (4, 8, 50), we addressed the possibility that our failure to detect increased E-selectin expression might be due to the lengthy induction time (16 h) which was used. We therefore used a short-term induction period in which endothelial cells were exposed to monocyte culture fluids for 3 h at 37°C prior to assay for E-selectin expression by ELISA. As shown in Fig. 3, increased E-selectin expression was observed in endothelial cells treated with culture fluids from monocytes inoculated with virus-antibody (1:640 and 1:2,560) combinations, i.e., similar to those shown above to be effective in inducing VCAM-1 and ICAM-1 expression. These combinations are also the ones determined to be most effective in enhancing dengue virus infection of monocytes (Fig. 1). In summary, therefore, the results indicate a correlation between the magnitude of dengue virus infection of monocytes and the production of endothelial cell-activating factor(s) in monocyte culture fluids. The resultant activation of endothelial cells is characterized by a fairly short-lived increase in E-selectin expression (i.e., evident at 3 h but not at 16 h of treatment), whereas elevated expression levels of VCAM-1 and ICAM-1 are detectable at 16 h of treatment.

**Requirement for infectious virus.** To determine whether infectious virus was required for the induction of endothelial cell-activating activity in monocytes, monocytes were inoculated with mixtures of antiserum and virus which had been inactivated by UV. As shown in Fig. 4, endothelial cell-activating activity was detected only in the culture fluid supernatants from monocytes infected with mixtures of dengue virus-immune serum and infectious virus. Virus which had been inactivated by UV, prior to mixing with dengue virus-immune serum, was unable to induce such activity in monocytes, suggesting that virus replication in monocytes is required for the induction of a endothelial cell-activating factor(s).

**Evidence that TNF-α is a major endothelial cell-activating factor induced by dengue virus infection of monocytes.** Monocytes secrete certain factors (e.g., TNF-α and IL-1β) which are known to activate endothelial cells (4, 8, 11). To determine whether such a factor might be responsible for the observed endothelial cell activation by culture fluids from dengue virus-infected monocytes, monocyte culture fluids were pretreated with anti-TNF-α antibody or IL-1RA. The treated and untreated culture fluids were then incubated for 16 h with endothelial cells, which were then assayed for ICAM-1 expression by ELISA. As shown in Fig. 5, culture fluid from monocytes inoculated with a combination of virus and 1:640 dilution of antiserum produced a strong increase in endothelial cell ICAM-1 expression (similar to that observed in Fig. 2). This increase was almost completely abolished when the monocyte culture fluid was pretreated with anti-TNF antibody (Fig. 5). A much smaller inhibition of increased ICAM-1 expression was observed after similar treatment with IL-1RA. For compari-
son, the increased ICAM-1 expression mediated by culture fluids from LPS-stimulated monocytes was mediated by a combination of TNF-α and IL-1, as indicated by partial blocking with anti-TNF-α and IL-1RA (Fig. 5). The results strongly implicate TNF-α as a major endothelial cell-activating factor produced by dengue virus-infected monocytes.

Lack of endothelial cell activation by direct infection of endothelial cells with dengue virus. Since endothelial cells can be infected by dengue virus (2, 30), it is important to establish whether such infection may provide a mechanism for endothelial cell activation. We inoculated parallel cultures of endothelial cells and Vero cells with dengue virus and with virus-antibody combinations. Virus-antibody combinations were used since it has been reported that certain types of endothelial cells possess Fc receptors (1, 46) which might conceivably enhance dengue virus infection. An MOI of 0.1, the same as that employed for the monocyte infections, was used. Concentrations of antibody used bracketed the range of concentrations shown above to be effective in enhancing dengue virus infection in monocytes (Fig. 1). Cultures were radiolabeled from 36 to 48 h postinoculation, and aliquots of the culture fluids were immunoprecipitated with dengue virus-immune serum. Analysis of the radiolabeled proteins present in the immunoprecipitates revealed abundant amounts of virus-specific proteins in the culture fluids from Vero cells but less than detectable amounts in culture fluids from endothelial cells. Thus, while endothelial cells can be infected by dengue virus (2, 30), the level of infection is far below that observed either in Vero cells or in blood monocytes (compare Fig. 1 and 6).

Examination by ELISA of adhesion molecule expression on endothelial cells directly inoculated with dengue virus showed no evidence that VCAM-1, ICAM-1, or E-selectin was increased in expression (Fig. 7). Taken together, the results suggest that the major mechanism of endothelial cell activation by dengue virus is an indirect one, requiring virus infection of monocytes and the production in infected monocytes of intermediary cytokines, notably TNF-α.

**DISCUSSION**

The results of this study provide evidence that dengue virus infection can activate endothelial cells, as indicated by increased expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin. The method of activation is primarily an indirect one, requiring the participation of virus-infected monocytes, rather than the direct infection by virus of endothelial cells themselves. This observation is consistent with histopathological studies which indicate low or nonexistent infection of endothelial cells in tissues from patients suffering from DHF/DSS (reviewed in references 15 and 16).

A number of viruses are associated with hemorrhagic disease, although the underlying mechanisms remain poorly un-
understood. Marburg virus, a filovirus, replicates efficiently in endothelial cells (comparable to the replication seen in monkey kidney cells) and produces a destructive infection (45) which appears to parallel the endothelial cell lysis observed in filovirus-infected animals (12). In contrast, the results of our present study indicate that the endothelial cell is not a major target of infection by dengue virus and that endothelial cell abnormalities arising from dengue virus infection are caused primarily by immunological mediators. This is not to say that under conditions of heavy virus load, endothelial cells are not infectable by dengue virus, but merely that when exposed to a given antibody-virus inoculum, monocytes are likely to be the major targets of infection and consequently the vasoactive effects on endothelial cells are likely to be mediated indirectly.

Dengue virus is unique among viruses in exploiting to a tremendous degree the phenomenon of antibody-dependent enhancement of infection. Infection of dengue virus in peripheral blood monocytes is increased dramatically in the presence of subneutralizing levels of virus-specific antibody (5, 6, 10, 18, 19, 38), thus permitting enhanced virus cell attachment via the monocyte Fc receptors (42). At MOIs of up to about 0.1, virus-specific antibody may in fact be essential for dengue virus replication in human monocytes (18). Such potential for virus-antibody complexes to selectively infect Fc receptor-bearing cells such as monocytes provides a unique opportunity for dengue virus to provoke a powerful response in these important orchestrators of immune function. The present study supports this hypothesis, by demonstrating that antibody-enhanced infection of monocytes by dengue virus triggers the production of endothelial cell-activating factor(s), particularly TNF-α. The observation that virus-antibody combinations are much more potent than virus alone in producing this activation shows an interesting parallel with known epidemiological evidence that preexisting virus-specific immunity is a powerful risk factor for DHF/DSS in human dengue virus infections (15).

This study may provide, in part, a mechanistic explanation for the elevated levels of TNF-α found in sera from dengue virus-infected patients, especially those suffering from DHF/DSS (22, 49, 51). The source of such TNF may be dengue virus-infected monocytes (this study), T cells which are acti-
vated during dengue virus infection (34), or a combination thereof. TNF-α has a variety of activities relevant to producing vascular abnormalities and is associated with other (nonviral) clinical conditions of shock (3, 11). TNF-α is known to also be a potent inducer of IL-6 in endothelial cells (28). IL-6 could play a major role in the pathogenesis of hemorrhaging and shock, due to its ability to mediate increased endothelial cell permeability (36). In addition, TNF-α is an activator of lipid mediator biosynthesis, including that of platelet-activating factor, leukotrienes, prostaglandins, and thromboxanes (35). Of these lipid mediators, platelet-activating factor is one of the most significant, due to its direct and potent action on vascular permeability (7) as well as its role as eicosanoid donor for vasoactive leukotriene and thromboxane biosynthesis (35). Thus, TNF-α is capable of initiating a cascade of processes which may contribute to endothelial dysfunction in DHF/DSS. Studies are in progress to elucidate further the pathological effects on vascular endothelial cells following exposure to the secreted products from dengue virus-infected monocytes.

It would be of interest to compare the effects of viruses other than dengue virus on monocyte production of endothelial cell-activating factors; however, the use of unrelated viruses as potential controls for study makes valid comparisons difficult. Viruses such as human immunodeficiency virus (HIV), influenza virus, and respiratory syncytial virus are able to infect blood monocytes and are susceptible to antibody-dependent enhancement of infection (13, 14, 24, 33, 40, 41, 43, 47, 48). However, influenza virus and respiratory syncytial virus are respiratory viruses which, unlike dengue virus, do not likely infect blood monocytes in vivo to any great degree; the relevance of in vitro studies of monocyte infection to natural pathogenesis therefore remains unclear. In the case of HIV, which does infect monocytes during natural infection (37), antibody-dependent enhancement rarely attains a 10-fold increase in infection (14, 24, 43, 47), whereas dengue virus infection is enhanced by antibody to much higher levels, generally within the range of 10- to 1,000-fold (5, 6, 10, 18, 19, 38). Enhancing antibody is therefore much less likely to be a discriminating factor in HIV pathogenesis than it is in severe dengue virus disease (15–17). Nevertheless, in the context of our present finding that TNF-α is produced by dengue virus-infected monocytes, it is interesting that HIV also induces TNF-α production in monocytes (44) and that the induced TNF-α, along with other cytokines and viral gene products, can modulate endothelial cell function (23, 44). In HIV infection, it is thought that such modulation of endothelial cell function may enhance transmission of virus and/or virus-infected cells through endothelial cell barriers (23, 39). In contrast to the generally slow and chronic nature of HIV infection, dengue virus is rapid and acute. In consequence, the dengue virus-associated cytokine responses (including the TNF-α response) and their effects on endothelial cell function are likely to be much more abrupt than those associated with HIV infection and may manifest themselves in more precipitous changes in hemostasis such as those associated with DHF/DSS.

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REFERENCES


