IL8 release, tight junction and cytoskeleton dynamic reorganization conducive to permeability increase are induced by dengue virus infection of microvascular endothelial monolayers

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Permeability alterations of microvascular endothelia may be a factor in the plasma leakage produced by dengue virus infection. Confluent monolayers of the human dermal microvascular endothelial cell line HMEC-1 were utilized as an experimental model to study the cellular responses induced by the virus. Infected monolayers showed increased permeability for [3H]mannitol, but no changes were observed for 4–70 kDa dextrans at 48 h post-infection (p.i.), a time at which viral titres reached maximal values and 40 % of the cells expressed viral proteins. A further increase in permeability occurred at 72 h, still without evident cytopathic effects on the monolayer. Coinciding with this, actin was reorganized in the infected cells and the tight junction protein occludin was displaced to the cytoplasm. Increments in the thickness of stress fibres and focal adhesions were observed in uninfected cells neighbouring infected cells. Culture medium from infected monolayers induced permeability changes and thickening of actin-containing structures in control cultures that resembled those observed 48 h p.i. Interleukin (IL) 8 was found in culture medium at concentrations ranging from 20 to 100 pg ml−1. Neutralizing antibodies against IL8 partially inhibited the changes produced by the culture medium as well as those induced by addition of IL8. Genistein inhibited the effect of the culture medium and the phosphorylation of proteins associated with focal adhesions and indicated the participation of tyrosine kinases. These findings suggest that IL8 production by infected monolayers contributes to the virus-induced effect on the cytoskeleton and tight junctions and thereby modifies transendothelial permeability.

INTRODUCTION

Dengue virus, a single-stranded RNA virus, causes the febrile, debilitating illness called dengue fever (DF) (Chambres et al., 1990). Some patients develop a more severe disease known as dengue haemorrhagic fever (DHF), in which the most relevant features consist of a drastic increase of endothelial permeability, thrombocytopenia and coagulation disorders (Rigau-Pérez et al., 1998). Endothelial permeability alterations can lead a patient to hypovolaemic shock, a situation called dengue shock syndrome (DSS), and death. The mechanisms associated with endothelial permeability increase are not well understood, nor is it known whether endothelial cells are altered by the viral infection. Virus entry is suspected as viral RNA has been detected in brain microvascular endothelial cells from a fatal case of DHF (Ramos et al., 1998). Interleukin (IL) 8 and other cytokines have been proposed as inducers of alterations in endothelial function, because elevated levels of cytokines have been found in serum and pleural fluid of patients with DHF and in dengue virus-infected human primary monocytes (Raghupathy et al., 1998; Bosch et al., 2002).

Analysis of dengue virus–endothelium interaction has been approached using human endothelial cells from primary cultures of umbilical cord (HUVEC) or cell lines derived from it (Andrews et al., 1978; Chen et al., 1996; Bosch et al., 2002). In these in vitro models it has been shown that the viral E protein binds to the endothelial cell surface in the absence of Fc receptors (Chen et al., 1996) and that infected cells produce high levels of IL8 as well as IL6 (Bosch et al., 2002; Huang et al., 2000). E protein also binds to other cell lines such as Vero, Chinese hamster ovary and glia (Chen et al., 1996).

The natural target for Dengue virus would be microvascular
endothelial cells in several tissues where plasma leakage is believed to occur (Krishnamurti et al., 2001; Jacobs & Levin, 2002). Because the specific response to virus penetration in these cells is still poorly understood, we have utilized human dermal microvascular endothelial cells (HMEC-1) to investigate the mechanisms by which the infection could induce cellular permeability changes. Compared with big-vessel endothelial cells (e.g. HUVEC), microvascular endothelial cells such as HMEC-1 could provide a suitable model to study dengue virus interaction, because this line constitutes a homogeneous population and forms stable monolayers that retain morphological and functional characteristics of normal human microvascular endothelia (Ades et al., 1992; Xu et al., 1994). Furthermore, these cells have well-organized tight junctions (TJ) and allow vectorial transport (Blum et al., 1997; Kielbassa et al., 1998). The TJ complex regulates permeability in epithelial and endothelial layers, acting as a selective barrier to passage of ions and other molecules (Anderson, 2001; Dejana et al., 2000). Several proteins participate in the organization and function of TJ. Importantly, their localization and interactions are modulated by association with the actin cytoskeleton, in particular the actin ring tethered to the plasma membrane on the apical side (Meza et al., 1980; Madara, 1991; Rao et al., 2002). An increase in cellular permeability could result from alterations in the sealing capacity of TJ, caused by modifications to the TJ complex interactions (Cereijido et al., 1989).

We report here that HMEC-1 can be infected by Dengue 2 virus (D2V) and that confluent monolayers formed by these cells show alterations in permeability at 48 h post-infection (p.i.), as well as actin cytoskeleton rearrangements and displacement of occludin from the TJ complex. These changes occur concomitantly with increases in the thickness of stress fibres and focal adhesions in uninfected cells in the same monolayer. Culture medium from monolayers, recovered 48 h p.i., also induced a significant increase in permeability and reorganization of actin-containing structures, including phosphorylation of tyrosines in proteins forming focal adhesions. These effects were closely reproduced by addition of IL8 to uninfected monolayers and were partially inhibited by neutralizing antibodies to IL8. Genistein, a specific inhibitor of phosphotyrosine kinases, inhibited the effects of the infected-cell culture medium. These data show that D2V infection of HMEC-1 monolayers induced an increase in endothelial permeability eliciting cytoskeleton rearrangements that facilitate TJ disorganization. Although studies in vitro cannot be extrapolated to the patient, our data suggest that endothelial alterations could occur in vivo after D2V infection. Mechanisms activated by secreted cytokines could balance the virus-induced alterations and possibly the secretion of anti-inflammatory cytokines by cells of the immune system, to regain tissue homeostasis and eliminate the virus (Chaturvedi et al., 1999). In the case of DHF, severe permeability alterations could be caused by similar mechanisms but inadequate T-cell activation, after a secondary infection, would contribute to the observed pathology, as proposed recently by Mongkolsapaya et al. (2003).

**METHODS**

**Cell lines and culture.** The HMEC-1 cell line was donated by E. W. Ades and F. J. Candal from the CDC (Atlanta, GA, USA) and T. J. Lawley (Emory University, Atlanta, GA, USA). These cells retain specific markers for microvascular endothelial cells and endothelial cell primary cultures (Ades et al., 1992; Xu et al., 1994). Confluent monolayers were grown at 37 °C under 5% CO2 in MCDB131 medium (Gibco-BRL, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 ng human epidermal growth factor ml-1 (Gibco), 1 μg hydrocortisone ml-1 (Sigma-Aldrich) and 100 U of penicillin and 100 μg of streptomycin per ml of culture medium (In vitro). Cells were used at passages 20 to 25. For propagation of D2V, C6/36 HT cells were used and BHK-21 (clone 15) were used for titration of virus infectivity. They were grown at 34 and 37 °C, respectively, in minimal essential medium (MEM) (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin.

**Preparation of virus stocks, titration and inactivation.** D2V was donated by B. Briseno (Institute for Diagnosis and Epidemiologic Reference, Mexico City). The sample was isolated from a Mexican patient who developed DF. Monolayers of C6/36 HT were inoculated with the virus and incubated at 34 °C for up to 12 passages. After the last passage, cells were lysed by freeze–thaw cycles and stored in aliquots at −70 °C. Virus was titrated by the plaque assay using BHK-21 cells. Briefly, a tenfold serial dilution of the virus was added to BHK-21 monolayers cultured in 24-well plates at 2·5·105 cells ml−1 and these were incubated at 37 °C for 4 h. After this time, 0·5 ml MEM containing 10% FBS and 3% (w/v) carboxymethylcellulose was added to each well. After 5 days incubation at 37 °C, plaques were visualized by staining with naphthol blue black. Virus concentrations are given as p.f.u. ml−1. Virus inactivation was performed as indicated by Anderson et al. (1997) by exposure to short-wave UV-light irradiation from a germicidal lamp for 10 min. The inactivation of virus infectivity was verified by plaque assays in BHK-21 cells.

**Cell infection.** Confluent monolayers of HMEC-1 were trypanocized and resuspended in growth medium. Cells were seeded on glass coverslips (3·5·105 cells ml−1) for immunofluorescence assays and on collagen-coated 12 mm Millicell filter inserts with a 0·4 μm pore size (Millipore) for permeability assays. After 72 h, the culture medium was removed from confluent monolayers and active or UV-inactivated virus was added at 1 p.f.u. per cell and incubated at 37 °C for 90 min. After this time, the inoculum was removed and fresh growth medium was added. Infected cells were analysed at 12, 24, 48, 72 and 96 h p.i.

**Detection of viral proteins E and NS1.** Virus-infected HMEC-1 monolayers grown on glass coverslips were fixed in 3·7% formaldehyde for 20 min and were then permeabilized for 5 min with 0·05% Triton X-100 in PBS. After rinsing with PBS, cells were blocked with PBS/2% BSA for 30 min and exposed for 1 h at 37 °C to mAbs to D2V E protein (donated by the Pedro Kouri Institute, Havana, Cuba) or NS1 protein (donated by A. Falconar, London School of Hygiene & Tropical Medicine, U.K; Falconar & Young, 1991) at 1:10 and 1:50 dilutions, respectively. After washing with PBS, cells were incubated with FITC-conjugated anti-mouse IgG at 1:200 dilution (Molecular Probes) for 1 h at 37 °C. Coverslips were mounted with VectaShield H-1000 (Vector Laboratories). Stained cells were analysed with a Zeiss epifluorescence microscope using a 63× Planapo objective.
Flow cytometry analysis. At 12, 24, 48, 72 and 96 h p.i., D2V-infected and control cells were harvested from six-well culture plates. For flow-cytometry determination of infection, the harvested cells were washed twice with MCDB131 and then fixed with 3-7 % formaldehyde in PBS for 20 min at room temperature. After rinsing with PBS, cells were permeabilized with 0-05 % Triton X-100 in PBS and incubated with the mAb to the D2V E protein at 1:50 dilution for 30 min at room temperature. Cells were washed twice with PBS and incubated with FITC-conjugated anti-mouse IgG for 30 min at room temperature. After rinsing with PBS, cells were analysed in a fluorescence-activated cell sorter (Becton Dickinson).

Permeability measurements. Permeability was determined by measuring the paracellular passage of different sizes of fluorescein isothiocyanate dextran (FITC–dextran) (Sigma-Aldrich) and [3H]mannitol (NEN Life Science Products). Cells were grown to confluency on filter inserts and infected at 1 p.f.u. per cell. [3H]Mannitol and 4, 70 and 500 mM FITC–dextran were dissolved in the medium and in Ringer’s buffer (115 mM NaCl, 25 mM NaHCO3, 5 mM K2HPO4, 2 mM MgSO4, 1 mM CaCl2 and 2 mM L-glutamine), respectively. To measure flux in the apical to the basolateral direction, the tracer solution [1-25 μCi ml–1 (46-25 kBq ml–1) for [3H]mannitol or 10 μg ml–1 for the FITC–dextran of different sizes] was loaded on the apical side of the monolayer and cells were incubated for 1 h at 37°C. After this period, the tracer concentration in the basolateral compartment was measured. Concentrations of [3H]mannitol were determined in a β-scintillation counter. The FITC–dextran concentration was determined using a spectrofluorometer at an excitation wavelength of 492 nm and emission at 520 nm.

Localization of actin filaments, occludin, vinculin and tyrosine-phosphorylated proteins. HMEC-1 were grown to confluency on glass coverslips and were treated with these culture media for 2 h. The flux of [3H]mannitol was measured in the monolayers grown on the filter inserts and cells on glass coverslips and were stained for actin, vinculin and phosphotyrosines, as described above.

Cytokine determination. IL8 was measured in culture medium from D2V-infected monolayers or from inactivated virus-inoculated monolayers by microELISA (Quantikine; R&D Systems). The minimum detectable concentration for IL8 by this assay was 10 ng ml–1. TNF-α activity was tested in a bioassay in which exposure to 10 ng TNF-α ml–1 induced cell death in more than 50 % of L929 cells within 12 h (Evans, 2000).

Culture medium from D2V-infected and inactivated-virus-inoculated cells. HMEC-1 were grown to confluency in 24-well tissue culture plates and were inoculated with active D2V or UV-inactivated D2V at 1 p.f.u. per cell. The inoculum was removed after 90 min and fresh medium was added. At 48 h p.i., culture medium was collected and exposed to UV light to inactivate contaminating viral particles, as described above. Medium was stored at 4°C under sterile conditions and used within 1 week. Confluent monolayers were grown on inserts or glass coverslips and were treated with these culture media for 2 h. The flux of [3H]mannitol was measured in the monolayers grown on the filter inserts and cells on glass coverslips were stained for actin, vinculin and phosphotyrosines, as described above.

Effect of IL8. Recombinant IL8 (Quantine Systems) was added to control confluent monolayers at concentrations of 100–200 pg ml–1 dissolved in MCDB131. Cells were treated for 2 h at 37°C and permeability measurements, actin, vinculin and phosphotyrosine staining were evaluated as described above.

Inhibition of IL8 effect. Culture media from D2V-infected cells and from inactivated D2V-inoculated cells were mixed with antibodies against IL8 (Santa Cruz Biotechnology) or with irrelevant antibodies at a final concentration of 5 μg ml–1 and incubated overnight at 4°C. Confluent monolayers were grown on glass coverslips and were treated with these culture media for 2 h. Permeability measurements and cell staining were done as described above.

Inhibition of protein phosphorylation and actin reorganization. Confluent HMEC-1 monolayers were grown on coverslips, serum-starved for 12 h and pretreated with 60 μg genistein ml–1 for 1 h (Sigma-Aldrich). The cells were then exposed to virus-infected cell-culture medium or culture medium from cells inoculated with inactivated D2V. After 2 h, cells were fixed and stained to visualize actin and phosphotyrosines.

Statistics. Data are expressed as mean ± standard error of three independent experiments. The statistical significance was assessed by Student’s t-test.

RESULTS

D2V generates a productive infection in HMEC-1

HMEC-1 is a cell line derived from primary cultures of dermis endothelial cells that have retained the features of an active transport endothelium (Ades et al., 1992; Kielbassa et al., 1998). Expression of viral proteins in these cell monolayers after infection with D2V was revealed by immunofluorescence. Fig. 1(a, b) shows that the E protein, forming the virus envelope, and the NS1 protein, involved in virus assembly or release, are present in infected cells, indicating virus penetration and assembly. Analysis by flow cytometry indicated that, at 12 h p.i., 1 % of the cells expressed the viral E protein; the percentage increased to 13 % at 24 h and reached a maximum of about 40 % by 48 h. Virus titres, determined as the number of p.f.u. in BHK-21 cells infected with virus isolated from HMEC-1-infected monolayers, corresponded to 4·4 × 104, 2·1 × 105, 2·3 × 105 and 2·2 × 105 p.f.u. ml–1 at 24, 48, 72 and 96 h, respectively. At 12 h detectable virus plaques were not formed.

Morphological changes induced by D2V

After 60 h in culture, HMEC-1 are confluent monolayers, formed by polyhedral cells in close contact with each other. Cells have different heights and diameters that can be seen in the phase-contrast micrograph shown in Fig. 2(a). After D2V infection, monolayers remained morphologically intact, with no evidence of cytopathic effect, for 24 and 48 h. Fig. 2(b) shows a monolayer at 48 h p.i., where the general aspect of the cells is very similar to that of cells in Fig. 2(a). At 72 h p.i., cells appeared less flat and their borders became more evident (Fig. 2c). Detachment from contiguous cells and from the substrate were only observed after 96 h (Fig. 2d). Matching photographs in the right-hand side of each figure show infected cells in the same
field, using antibodies against the D2V E protein. Infected cells are seen forming clusters scattered in the monolayer.

Permeability alterations induced by D2V

Transendothelial permeability measurements in primary cultures of human microvascular endothelial cells have indicated that these cells may form more intact, regulatable TJ than other endothelial cell types (Blum et al., 1997). As shown in Fig. 3(a, b and c), HMEC-1 confluent control or inactivated-virus-inoculated monolayers do not permit the passage of even small molecules at any time of culture. However, at 48 h p.i. – when no cytopathic effect was evident by phase-contrast microscopy but immunofluorescence for viral antigen indicated that 40% of the cells were infected – a tenfold increase in permeability for $[^{3}H]$mannitol was observed in infected monolayers (Fig. 3a), while no permeability changes were registered at this time for 4, 70 and 500 kDa FITC–dextran (Fig. 3b). At 72 h, a significant increase in permeability was registered for all different sizes of FITC–dextran in the infected monolayers, indicating loss of the endothelial barrier function (Fig. 3c), although, at this time, the percentage of infected cells was not much higher than that observed at 48 h. In contrast, at 96 h, as expected from the morphological changes observed in Fig. 2(d), the endothelial monolayer showed a drastic increase in permeability to all the molecules tested (data not shown).

Actin cytoskeleton rearrangements and occludin redistribution induced by D2V

It has been shown that disarray of the actin ring tethered to the plasma membrane in the apical side of epithelial and endothelial cells disrupts the organization of the TJ complex and the permeability of confluent monolayers (Meza et al., 1980; Lum & Malik, 1996). Infected HMEC-1 showed disorganization of actin structures and the actin ring. Fig. 4(a, c and e) shows that, at 24 h p.i., cells expressing the viral E protein also contain disorganized actin, while no alterations of the actin ring or stress fibres were observed in adjacent uninfected cells. At 48 h p.i., actin disorganization became more evident in infected cells (Fig. 4b, d). Surprisingly, at this time, stress fibres located in the basal side of uninfected cells appeared more numerous, thicker and better organized (Fig. 4b, f). Longer times of infection (72 and 96 h) produced progressive disorganization of the actin pattern, as the infected monolayer showed further morphological alterations (data not shown).

Localization of the TJ protein occludin and its interaction with the TJ complex are stabilized by the actin cytoskeleton (Furuse et al., 1993; McCarthy et al., 1996). Therefore, as permeability and actin organization were perturbed by D2V infection, the distribution of occludin in HMEC-1 was analysed. Immunostaining of occludin and comparison between control and infected monolayers showed that this protein, which is normally localized following the contour of the TJ at the cell periphery as a continuous line, became discontinuous in D2V-infected cells. Fig. 5(a) shows that occludin in control cells is localized as a bright continuous line. As cells in a monolayer are not all in the same focal plane because of different heights and shapes, and epifluorescence images capture total volumes, slight fluorescence is observed in the cytoplasm of some cells. Fig. 5(b) shows infected cells 48 h p.i., where occludin staining follows the contour of two focused cells while, in others, discontinuity of the bright line can be seen and occludin aggregates appear in the cytoplasm. The discontinuity of occludin increased at 72 h p.i. and cells showing occludin aggregates in the cytoplasm became more numerous (Fig. 5c). By 96 h, occludin aggregates and increased fluorescence in the cytoplasm suggest that the protein is being removed from the cell membrane (Fig. 5d). These changes were related in time to the gradual increase in permeability and the disorganization of the actin caused by virus infection, as shown in Figs 3 and 4.
D2V-infected cell-culture medium and IL8 induce alterations in the actin cytoskeleton and focal adhesions of control monolayers

Actin reorganization observed in non-infected cells neighbouring infected ones in the same monolayer after 48 h of infection suggested the presence of chemical mediators such as IL8 and TNF-α, because these cytokines have been proposed to be inductors of actin rearrangements (Blum et al., 1997; Schraufstatter et al., 2001). Furthermore, levels of IL8 have been found to be elevated in sera and pleural fluid of patients with DHF (Raghupathy et al., 1998), and it has also been detected in DV-infected HUVEC (Bosch et al., 2002). MicroELISA assays revealed the presence of increasing concentrations of IL8 in the culture medium of virus-infected monolayers. The concentration ranged from 20 pg ml⁻¹ at 24 h to approximately 100 pg ml⁻¹ at 48 and 72 h p.i. The presence of TNF-α was not detected at any time p.i. nor was IL8 found in the culture medium of uninfected monolayers or monolayers inoculated with inactivated virus. To evaluate the possible effects of D2V-infected cell-culture

Fig. 2. Morphological changes in HMEC-1 confluent monolayers as a function of infection time. (a) Uninfected monolayer; (b–d) infected monolayers at 48 (b), 72 (c) and 96 (d) h p.i. Phase-contrast microscopy shown to the left and the matching figures to the right show the corresponding immunofluorescence using the antibody to D2V E protein. Bar, 100 μm.
medium on the actin rearrangements observed in infected monolayers, confluent control monolayers were incubated for 2 h with medium obtained from cultures infected for 48 h with virus treated with UV light to eliminate infectious viral particles and with medium from monolayers inoculated with inactivated D2V. Staining for actin and vinculin, utilizing fluorescent phalloidin and vinculin antibodies, revealed that, in contrast to typical actin rings at the apical level and thin stress fibres and sparse focal adhesions at the basal level, present in cells in control monolayers (Fig. 6a, b), treatment with D2V-infected cell-culture medium induced the formation of thicker actin stress fibres and numerous focal adhesions (Fig. 6c, d). Addition of IL8, also for 2 h, to uninfected monolayers induced similar modifications in stress fibres and focal adhesions (Fig. 6e, f), suggesting that reorganization of the cytoskeleton could be at least partially elicited by the presence of IL8 in the culture medium of virus-infected monolayers.

Permeability alterations and actin rearrangements induced by D2V-infected cell-culture medium and IL8

When HMEC-1 confluent monolayers were treated for 2 h with medium from cells infected for 48 h and the permeability to $[^3]$Hmannitol was measured, an approximately fivefold increase was registered compared with the flux in uninfected monolayers (Fig. 7a). To test whether IL8 activity could be responsible for the effects elicited by infected-cell culture medium, IL8 was added for 2 h to uninfected monolayers and the permeability to $[^3]$Hmannitol was measured. A threefold increase was observed, with concentrations of IL8 ranging from 100 to 200 pg ml$^{-1}$. Neutralizing antibodies to IL8 added to culture medium from infected monolayers partially, but significantly, inhibited the increase in permeability elicited by the culture medium. Furthermore, IL8 incubated previously with the antibodies and then added to uninfected monolayers did not alter permeability. No effects on permeability were observed using an irrelevant antibody with the same isotype.

The inhibitory action of antibodies to IL8 on actin reorganization induced by the cytokine or by virus-infected cell culture medium is shown in Fig. 7(b–e). Fig. 7(b, c) shows the arrangement of actin fibres in treated monolayers. These changes were not induced when antibodies to IL8 were present in culture medium from non-infected cells containing IL8 or in medium from infected-cell cultures (Fig. 7d, e). These data further support the participation of IL8 in the reorganization of actin-containing structures.

Fig. 3. Permeability changes in HMEC-1 confluent monolayers after infection with D2V. (a) Permeability to $[^3]$Hmannitol, 48 h p.i. *, $P<0.001$. (b) Permeability to different sizes of FITC–dextran, 48 h p.i. (c) Permeability to different sizes of FITC–dextran, 72 h p.i. *, $P<0.001$. In all experiments shown, monolayers grown in uninfected cell culture medium are identified as Control, monolayers inoculated with UV-light-inactivated D2V as iD2V and monolayers infected with active D2V as aD2V. The permeability of the filter without cells was used as reference (Filter).
Participation of phosphotyrosine kinases in actin rearrangements induced by D2V-infected cell-culture medium

The cellular mechanisms by which IL8 could be inducing changes in the actin cytoskeleton were explored considering the reported role of cytokines in signalling pathways, and in particular of IL8 as an effector for Rho and Rac GTPases (Schraufstatter et al., 2001). Therefore, the participation of phosphotyrosine kinases (PTK) in the modification of stress fibres and focal adhesions in monolayers treated with D2V-infected cell-culture medium or IL8 was analysed. A strong

**Fig. 4.** Double-staining of infected HMEC-1 monolayers to visualize the viral E protein and actin at 24 (a, c and e) and 48 h p.i. (b, d and f). Cells were stained with rhodamine–phalloidin (a and b) and with antibody to D2V E protein (c and d); superimposed images of the above figures are shown in (e and f). Infected cells are marked with asterisks in (a), (b), (e) and (f). The same cells contain the viral E protein in (c) and (d). Arrows point to stress fibres. Bar, 50 μm.
positive signal for phosphotyrosines was found in the numerous focal adhesions of cells treated with the D2V-infected cell-culture medium (Fig. 8c), which also showed thick and abundant stress fibres (Fig. 8a). Reduction in stress fibres and focal adhesion was observed when cells were pretreated for 1 h with genistein, before addition of culture medium from infected monolayers (Fig. 8b, d). Identical results were obtained in monolayers treated with IL8 after 1 h preincubation with genistein (data not shown). These results indicate that phosphorylation of tyrosines is necessary in proteins participating in actin reorganization at the basal level of the cells, suggesting that IL8 present in culture medium from virus-infected monolayers could induce their phosphorylation.

**DISCUSSION**

Dengue virus infection of human endothelial cells was proposed in 1978 (Andrews et al., 1978). Structural alterations of microvascular endothelial cells were found in skin biopsies and viral antigens were detected in microvascular endothelial cells from the brain of a fatal case of DHF (Sahaphong et al., 1980; Ramos et al., 1998). Not having access to animal models to reproduce the disease, knowledge about its physiopathology has been mostly inferred from in vitro studies utilizing endothelial cell lines derived from big vessels such as the umbilical cord vein, such as HUVEC. Although these cells can be infected by Dengue virus (Andrews et al., 1978; Chen et al., 1996; Bonner & O'Sullivan, 1998; Huang et al., 2000), the transendothelial barrier function of cultured HUVEC monolayers shows great fluctuations depending on culture conditions, making them an inadequate system to assess endothelial permeability alterations expected to be induced by the virus (Bonner & O'Sullivan, 1998; Jacobs & Levin, 2002). On the other hand, it is known that the microvascular endothelium, especially in post-capillary venules, develops tighter barriers to passage of macromolecules, it is the target of many chemical mediators, it responds differently to chemical stimuli than do macrovascular endothelial cells and it is the site where plasma leakage may occur as a result of permeability increase (Del Vecchio et al., 1992; Blum et al., 1997).

The HMEC-1 line, derived from human dermis microvasculature, retains morphological, biochemical and functional characteristics of primary microvascular endothelial cultures and forms TJ complexes that maintain a stable barrier function. Therefore, these cells offer an in vitro model with closer similarity to the tissue that seems to be the main target of pathogens such as Dengue virus (Ades et al., 1992; Xu et al., 1994; Kielbassa et al., 1998). We report here permissiveness of HMEC-1 monolayers to productive...
infection by D2V. At 48 h p.i., and before any evident cytopathic effect, permeability changes were detected for small molecules such as $[^3H]$mannitol, suggesting a slight modification of the TJ function as a permeability barrier. The barrier function to macromolecules was lost at later times of infection (72 h), although cytopathic effects, manifested as cell rounding and detachment from the substrate, were evident only after several days.

The barrier function of endothelium depends on structural integrity and it is regulated by multiple factors (Lum & Malik, 1996; Blum et al., 1997). In HMEC-1 monolayers,
discontinuity in the localization of the transmembrane TJ protein occludin at the TJ complex region was observed 48 h p.i. with D2V. Loss of occludin continuity at the cell periphery coincided with permeability to mannitol and, at later times of infection, with a progressive increase in the permeability to larger molecules. Other viruses and some bacteria have been reported to alter the organization of TJ in host cells, but the mechanisms utilized by these pathogens are not yet fully understood (Cudmore et al., 1997; Obert et al., 2000; Amieva et al., 2003).

An important modulator of endothelial and epithelial permeability is the actin cytoskeleton through its interaction with TJ complex components (Meza et al., 1980; Madara, 1991; Lum & Malik, 1996; Blum et al., 1997). In dengue virus-infected HMEC-1, actin fibres forming the peripheral ring at the apical side were rapidly disorganized and possibly fragmented as actin aggregates were observed in the cytoplasm of infected cells. However, virus entry and replication did not drastically affect the sealing of TJ. Later, when viral particles were assembled and released, more dramatic modifications of the cytoskeleton coincided with displacement of occludin from the membrane and its concentration into aggregates and a significant increase in permeability. At the same time, thickening of stress fibres and focal adhesions was observed in uninfected cells neighbouring infected ones. The structural modifications of the cytoskeleton in uninfected cells cannot be explained by a direct effect of the virus as seems to be the case for infected cells but, rather, is interpreted as the result of a secondary effect of viral infection. It is possible that rearrangement of actin-containing structures into thicker stress fibres and focal adhesions will provide better attachment to the substrate to maintain the integrity of a monolayer threatened by viral infection.

It has been reported that cytokines modulate endothelial permeability and generate subtle changes in actin cytoskeleton organization and in the proteins forming intercellular junctions (Blum et al., 1997; Jiang et al., 1999; Schraufstatter et al., 2001). It is also known that HUVEC infected by Dengue virus release IL8 and other cytokines (Avirutnan et al., 1998; Bosch et al., 2002). However, reports of cell infection with Dengue virus have not addressed the role of cytokines on the structural reorganization of the actin cytoskeleton and its possible relationship with permeability changes. We report here that D2V-infected HMEC-1 cultures release IL8 and that addition of culture medium obtained 48 h p.i., or addition of IL8 to control monolayers, reproduced the modifications in permeability and reorganization of the cytoskeleton produced by infected cell culture medium. The partial inhibition of both permeability and actin reorganization by antibodies against IL8 corroborated the participation of this cytokine in the structural and functional modifications produced by virus-infected cell culture medium. However, the changes observed cannot be attributed to IL8 alone, because infected-cell culture medium showed stronger effects and antibodies to IL8 did not completely reverse its effects. Identification of other components in the culture medium of infected monolayers will be necessary before drawing definitive conclusions.

Our results also show that actin reorganization induced by viral infection was sensitive to inhibitors of PTK. This
finding is very suggestive of a role for IL8, and possibly other cytokines released by the infected endothelial cells, as activators of PTK signalling pathways, which, by phosphorylation of specific targets, could induce structural reorganization of TJ and actin (Lum & Malik, 1996; Schraufstatter et al., 2001). Previous reports regarding the disruption of TJ caused by elevated levels of tyrosine phosphorylation of proteins at the intercellular junction complex support this idea (Collares-Buzato et al., 1998; van Nieuw Amerongen et al., 1998). Furthermore, cell retraction as a consequence of TJ disarray and detachment from neighbours could also be regulated by PTK phosphorylation of actin-binding and TJ-associated proteins (Collares-Buzato et al., 1998; Chen et al., 2002). Activation of Rho family GTPases, which are the main participants in stress fibre formation and vinculin association with actin, could also be the consequence of higher levels of IL8 and other cytokines (Lum & Malik, 1996; Imamura et al., 1998). Very recently, it has been reported that IL8 induced tyrosine phosphorylation of the focal adhesion kinase (FAK) and modified its association with vinculin in focal adhesions (Feniger-Barish et al., 2003).

In summary, utilizing HMEC-1 monolayers as a model, we have shown that dengue virus entry and assembly can proceed in cells from the microvasculature. Virus infection can be correlated with TJ and actin cytoskeleton functional and structural alterations that lead to important increments in transendothelial permeability. Furthermore, virus infection elicits the release of IL8, which induces further structural modifications that may be important for dynamic changes in the monolayer. While studies in vitro with Dengue virus cannot be extrapolated to what occurs in the infected patient, our studies suggest that, in vivo, similar structural changes and release of cytokines by endothelial and other cells infected by the virus could elicit permeability alterations but, at the same time, activate mechanisms to maintain homeostasis and balance inflammatory responses to get rid of the virus and damaged cells (Carr et al., 2003). In more severe forms of the infection, such as DHF, the host organism may not be able to balance the effects of the virus and the inflammatory responses by primed lymphocytes and regain homeostasis, leading to drastic pathological manifestations, as recently suggested by Mongkolsapaya et al. (2003).

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**Fig. 8.** Participation of PTK in actin rearrangements induced by infected-cell culture medium and IL8. (a) Cells treated with culture medium from infected cells. (b) Cells preincubated for 1 h with 60 μg genistein ml⁻¹ and treated for 2 h with culture medium from infected cells in the presence of genistein. (c) Same cells as in (a) stained with antibody to phosphotyrosines. (d) Same cells as in (b) stained with the antibody to phosphotyrosines. Arrows point to stress fibres. Bar, 50 μm.
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