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Journal of Virological Methods 104 (2002) 173–185

Journal of Virological Methods

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An improved endothelial barrier model to investigate dengue haemorrhagic fever

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Received 22 January 2002; received in revised form 21 March 2002; accepted 21 March 2002

Abstract

A cell culture model suitable for studies of dengue haemorrhagic fever was developed, based on culture of primary human umbilical vein endothelial cells (HUVECs) on a permeable membrane. By electron microscopy, cultured HUVECs at day 11 resembled morphologically microvascular endothelium. Endothelial barrier function was assessed by measuring transendothelial flux of albumin. Instead of using a labelled tracer molecule, an enzyme-linked immunosorbent assay (ELISA) was developed to measure concentrations of native human albumin. The permeability characteristics of the HUVEC monolayer were found to be improved significantly (approximately 1 log reduction in permeability coefficient for albumin) by culturing HUVECs in human serum rather than fetal calf serum. Permeability coefficients for albumin in the range $1–4 \times 10^{-7}$ cm/s were achieved, which is an improvement on previous in vitro models of the endothelial barrier. Comparison of transendothelial flux of albumin and urea provided evidence of molecular sieving by the HUVEC monolayer. Moreover, tumour necrosis factor-α induced a dose-dependent, reversible increase in permeability of the HUVEC monolayer. This endothelial barrier model thus has many important characteristics that resembled human microvascular endothelium and is an improvement on the previous model proposed for studies of dengue haemorrhagic fever.

Keywords: Dengue; Endothelium; Permeability; Tissue culture; Albumin

1. Introduction

Dengue viruses are mosquito-borne flaviviruses that cause most commonly a benign febrile illness (dengue fever) and less frequently cause a life-threatening illness characterized by disordered haemostasis and increased vascular permeability (dengue haemorrhagic fever). In recent years, the geographical range of dengue in tropical and sub-tropical regions of the world has extended and dengue haemorrhagic fever is occurring in new areas of the world and with increased incidence

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0166-0934/02/$ - see front matter
PII: S0166-0934(02)00065-4

(Rico-Hesse et al., 1997). There are an estimated 100 million dengue virus infections worldwide each year (Halstead, 1988) and approximately 500,000 cases of dengue haemorrhagic fever are notified officially each year, although the true incidence is probably much higher.

Peak viraemia titre early in the course of infection correlates with disease severity in humans (Vaughn et al., 2000). It follows that host and viral factors that add to or subtract from the success of viral replication are critical determinants of disease outcome. There is considerable evidence that pre-circulating *anti*-dengue antibody is the predominant risk factor predisposing individuals to dengue haemorrhagic fever, via antibody-dependent enhancement of infection (Halstead, 1988). Particular viral strains are associated with severe clinical disease (Rico-Hesse et al., 1997; Watts et al., 1999) but the molecular basis of this variation in viral virulence is unknown.

Despite evidence that higher viraemia titres are associated with more severe disease, the molecular mechanisms underlying the pathogenesis of dengue haemorrhagic fever remain unknown. Vascular leak is the hallmark of dengue haemorrhagic fever. Vascular leak implies damage to the vascular endothelium, which constitutes the major permeability barrier in the vessel wall (Ramirez et al., 1984). A key property of the endothelial barrier is molecular sieving: water and microsolutes can exchange freely across the microvascular endothelium, whereas movement of large molecules such as albumin is restricted (reviewed in Michel, 1996, 1998). Molecular sieving by the vascular endothelium is essential for maintenance of a greater protein concentration in the plasma than in interstitial fluids. This, in turn, generates the osmotic pressure that retains the fluid components of blood within the vessels.

The clinical features of dengue haemorrhagic fever provide important clues to the nature of endothelial injury. Rapid resolution of vascular leak if the patient is supported adequately during the acute phase implies that derangement of the normal regulatory function of endothelial cells, rather than endothelial cell death, underlies vascular leak in dengue haemorrhagic fever. This is supported by biopsy and autopsy studies, which do not reveal morphological changes in endothelial cells, loss of vascular integrity nor infiltration of inflammatory cells into blood vessel walls (Halstead, 1989). It is believed widely that vascular leak is caused by soluble mediators of inflammation released as a consequence of immune responses to dengue infection. However, in the absence of an animal model of dengue haemorrhagic fever, it has been difficult to investigate the mechanisms of endothelial injury. One approach is to use cell culture systems to explore the link between dengue-infected cells and altered endothelial cell function. Using this approach, Anderson et al. (1997) showed that cultured human endothelial cells were activated, as demonstrated by up-regulation of adhesion molecules, when exposed to culture fluids from dengue-infected peripheral blood monocytes. Furthermore, inhibition of TNF-α abrogated most of this effect, suggesting that it is a key mediator in this system. However, the cell culture system used by Anderson et al. (1997) was not designed to assess endothelial barrier function. Bonner and O'Sullivan (1998) described an endothelial barrier model for the study of vascular leak in dengue haemorrhagic fever, but this model appears to be a poor approximation to the endothelial barrier in vivo (see Section 4). An improved in vitro model of the vascular endothelium was therefore developed that is well characterized physiologically and morphologically and is suitable for studying the mechanisms underlying vascular leak in dengue haemorrhagic fever.

2. Materials and methods

2.1. Isolation of endothelial cells

Primary human umbilical vein cells (HUVECs) were prepared from umbilical cord veins essentially as described by Jaffe et al. (1973). Cells isolated from individual umbilical cords were grown in a 25 cm^2 tissue culture flask in DMEM without HEPES (Life Technologies) with L-glutamine (1.6 mM), penicillin (80 i.u./ml), streptomycin (80 mg/ml) and 20% heat-inactivated (HI)

fetal calf serum (FCS, Life Technologies) at 37 °C in 5% CO_2. In some experiments, 20% pooled, heat-inactivated human serum (HS) (see below) was substituted for FCS, but many HUVEC cultures were then lost due to overgrowth of fibroblasts and this was abandoned. HUVECs in primary culture that failed to reach subconfluence within 24 h were discarded. All subsequent experiments were performed using first subculture HUVECs from a single donor, passaged from the primary culture using trypsin/EDTA (Sigma). Cells were verified as endothelial by morphology and the presence of Von Willebrand factor.

2.2. HUVEC growth on permeable membranes

HUVEC monolayers were cultured on Transwell Clear cell culture inserts (6.5 mm diameter, 0.4 μm pore size, Costar). These inserts hang in the wells of 24-well plates, creating a two-chamber culture system separating a luminal compartment (inside the insert) from a subluminal compartment (the well of the tissue culture plate). Prior to seeding of HUVECs, the membrane was coated with 100 μl 10% gelatin (Sigma) and then air-dried in a laminar flow cabinet overnight. The gelatin-coated inserts were then baked at 75 °C for 1 h. In some experiments, 0.3% type I collagen (ICN) was substituted for gelatin, in which case the baking step was omitted. Coated inserts were pre-conditioned by adding 200 μl cell culture medium (see below) to the luminal compartment and incubating for 4 h at 37 °C in 5% CO_2. The medium was then tipped off and the empty inserts were placed into wells containing 1 ml pre-warmed cell culture medium. Two hundred microlitre HUVEC suspension (5×10^5 cells/ml) in cell culture medium was added to each insert. Cells were cultured at 37 °C in 5% CO_2 for up to 21 days prior to measurement of transendothelial albumin flux. Cell culture medium in both chambers was replaced with fresh medium every 3 days.

2.3. Human serum

Human serum was separated from blood from 10 healthy, non-smoking volunteer donors. Whole blood was clotted on glass at 37 °C, then separated by centrifugation at $1200 \times g$ at room temperature for 10 min. Serum was collected, 0.2 μm filtered, HI (56 °C for 30 min), then stored in pooled aliquots at −70 °C.

2.4. Cell culture medium

HUVEC were grown in RPMI 1640 without HEPES (Life Technologies) with L-glutamine (1.6 mM), penicillin (80 i.u./ml), streptomycin (80 mg/ml) and either 20% heat-inactivated FCS or 20% pooled, heat-inactivated HS. In preliminary experiments, it was found that inclusion of 1% endothelial cell growth factor supplemented with heparin and heparin-binding growth factor-1 (Sigma) was essential for prolonged culture of HUVEC monolayers, and so this was routinely used. Medium without endothelial cell growth factor was substituted 24 h prior to measurement of transendothelial albumin flux.

2.5. Morphology of HUVECs

The morphology of HUVECs growing on the permeable supports was assessed by transmission electron microscopy (EM). Cells were gently washed with PBS, and then fixed in 0.5% gluteraldehyde in 0.1 M sodium cocodylate, pH 7.3 containing 0.5% alcian blue. All subsequent steps were performed at 4 °C. After washing in Ringer–Locke solution, cells were fixed in 1% gluteraldehyde in 0.1 M sodium cocodylate, pH 7.3 for 30 min. Cells were washed in 0.1 M sodium cocodylate, pH 7.3. Cells were then incubated in 1% tannic acid in 0.1 M sodium cocodylate, pH 7.3 for 5 min, followed by 1% osmium tetroxide in the same buffer for 30 min. Cells were washed in distilled water, and then taken through a graded series of ethanol washes (high grade ethanol, James Burroughs). The tissue culture inserts bearing the cells were then immersed in propylene oxide for 5 min, twice, then for 15 min. In this solution, the membrane with cells attached detached from the support, which dissolved. The cells were rinsed twice with propylene oxide, then incubated in propylene oxide/Araldite resin (50/50 v/v) mix for 30 min. After incubating twice in Araldite resin mix for 30 min, cells were baked for

48 h at 60 °C. Fifty to hundred nanometer thick sections were then cut using an ultramicrotome (Reichert), and transferred on to carbon formvar grids. Cells were incubated with 2% aqueous uranyl acetate for 5 min, then Reynold's lead citrate for 3 min. Cells were washed in distilled water, and observed using an accelerating voltage of 60 kV in a Jeol 100CX transmission electron microscope.

2.6. Exposure of HUVEC monolayers to TNF-α

In some experiments, HUVEC monolayers were exposed to various concentrations of recombinant human TNF-α (rTNF-α, Boehringer Mannheim) in complete culture medium for 24 h prior to measurement of transendothelial albumin flux. Unexposed HUVEC monolayers were always included as a control. In some experiments, heat-inactivated (90 °C for 15 min) rTNF-α was included as a negative control (Beynon et al., 1993).

2.7. Albumin flux across the HUVEC monolayer

Transwell inserts bearing HUVEC monolayers were washed gently by transferring them with sterile forceps through a sequence of wash baths containing pre-warmed RPMI 1640. Inserts without cells were always included to enable calculation of the permeability of the endothelial cell monolayer without the contribution of the coated membrane (see below). All experiments were performed in triplicate. After three washes, the inserts were placed in a fresh 24-well plate containing 1 ml pre-warmed RPMI 1640 without serum in each well. Next, 200 μl pre-warmed RPMI 1640 containing 40% heat-inactivated, pooled HS was added to each insert. The pooled HS contained a known quantity of albumin (46 mg/ml). The plate was then incubated at 37 °C in 5% CO_2 for exactly 4 h on an orbital shaker (4 mm orbit) at 180 rpm, which caused visible stirring of the chambers. At the end of the incubation period, the medium in each well was collected for measurement of albumin concentration (see below). In some TNF-α stimulation experiments, the inserts were then transferred to a fresh plate containing cell culture medium and, after a recovery period of 24 h, transendothelial albumin flux was measured again.

2.8. Measurement of albumin concentrations

A sandwich enzyme-linked immunosorbent assay (ELISA) was developed to measure native albumin concentrations in HS. ELISA plates ('High-bind', Greiner) were coated overnight with 100 μl mouse *anti*-human albumin mAb (A-6684, Sigma) diluted 1:10000 in carbonate buffer, pH 9.6. The plate was then washed four times in PBS/0.05% Tween 20 (PBS.T). The plate was next blocked with 200 μl 1% casein (Sigma) for 1 h. After repeating the wash step, 100 μl test samples diluted in PBS.T were incubated in the plate for 2 h. Samples were plated in duplicate. A standard curve, comprising doubling dilutions of serum containing a known albumin concentration, was included in duplicate on each plate. The plate was washed as before, then 100 μl sheep *anti*-human albumin-horseradish peroxidase conjugated polyclonal Ab (PP032, Binding Site) diluted 1:10000 in PBS.T was added to each well. After incubation for 1 h, the plate was washed as before and then developed by addition of 100 μl TMB substrate (K-blue, ELISA Technologies) to each well. The reaction was stopped by addition of 100 μl 2 M H_2SO_4 and the optical density at 450 nm of each well in the plate was read in an automated ELISA plate-reader (Molecular Devices Corporation).

2.9. Calculation of endothelial permeability

Permeability coefficients (PC) of the HUVEC monolayers, corrected for the contribution of the membrane, were calculated as described by Casnocha et al. (1989). In order to take account of the biological variability in baseline PC of HUVECs cultured from different donors (see Section 4), the effects of TNF-α exposure were calculated as ratios of exposed to unexposed PC using cells from a single donor. These ratios were then $\log_{10}$ transformed in order to enable normal distribution statistics to be used. The one-sample *t*-test was used to determine the statistical significance of the effects of TNF-α exposure.

2.10. Calculation of molecular sieving by HUVEC monolayer

In order to assess molecular sieving by the HUVEC monolayers, transendothelial flux of a small molecule, urea (MW 60; molecular radius 0.22 nm), was compared with albumin (MW 65,000; molecular radius 3.5 nm). Urea is present in HS and therefore the standard experimental protocol to measure transendothelial albumin flux was used to measure urea flux simultaneously. Urea concentrations were measured using a commercial enzymatic assay according to the manufacturer's protocol (BUN Endpoint, Sigma).

3. Results

Several simple models of the vessel wall based on culture of endothelial cells on permeable membranes have been reported previously. However, there is wide variation in the published methodology and a lack of consistent and comparable results. In this study, a systematic approach was taken to develop a model suitable for the study of dengue haemorrhagic fever and to optimize its morphological and physiological characteristics.

3.1. HUVEC culture and morphology

HUVECs formed confluent monolayers on the permeable membranes within 24 h, as judged by phase-contrast microscopy. In the presence of endothelial cell growth factor, these monolayers appeared to remain intact for >21 days in culture. During preparation for EM, it became apparent that HUVECs grew extensively up the inner surface of the luminal chamber, forming a cylinder of cells. This observation provided reassurance that permeability measurements in the model would not be confounded by an edge effect, which occurs if there are gaps at the edge of the cell monolayer on the membrane.

EM sections were performed on days 4, 11 and 21 of culture. At all time points, a continuous endothelial cell layer was observed and no intercellular gaps were identified in any section. The surface glycocalyx stained with alcian blue was visible as a hazy layer covering the endothelial cell surface (Fig. 1). After long culture periods (day 11 or 21), HUVECs exhibited the typical cross-sectional morphology of microvascular endothelium (Simionescu and Simionescu, 1984): cells were flattened, with a distinct bulge at the nucleus and short, well-defined intercellular junctions (Fig. 1). In contrast, after a short time in culture (day 4) the endothelial cell morphology was atypical: the cytoplasm was not flattened away from the nucleus and the intercellular junctions were long (Fig. 1). Huber and Weiss (1989) reported that HUVECs cultured

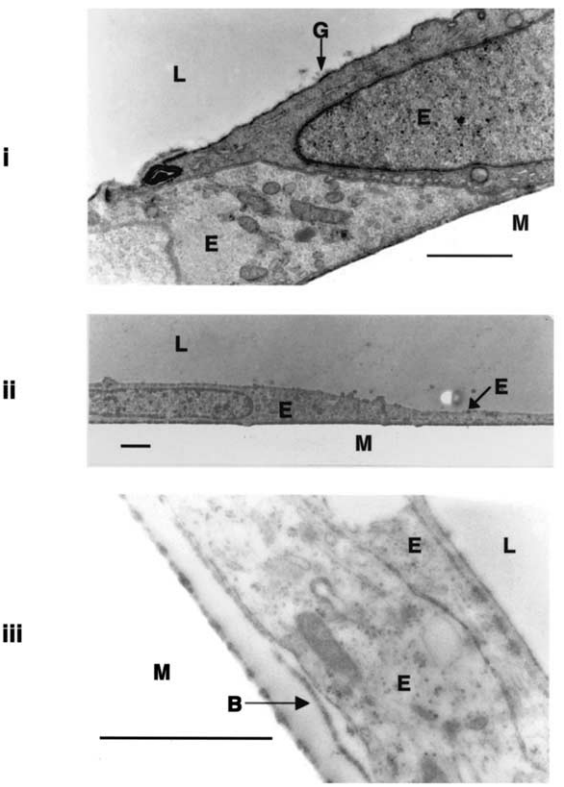

Fig. 1. Transmission electron micrographs of HUVEC cultures. Cultured endothelial cell monolayers were prepared for EM on day: (i) 4; (ii) 11; and (iii) 21. E, endothelial cell; B, basement membrane; G, surface glycocalyx (stained with alcian blue); L, luminal compartment; M, transwell membrane. The scale bar = 1 μm.

for 21 days on a type I collagen matrix secreted a continuous basement membrane. However, regardless of whether cells were grown on gelatin or collagen, the HUVEC cultures deposited only small, discontinuous lengths of basement membrane after 21 days (Fig. 1). Type I collagen offered no advantages in terms of morphology or function when compared with gelatin (data not shown), so that its use was abandoned in subsequent experiments. After 21 days in culture, HUVECs were beginning to overlap so that in some places the cell layer was two cells thick (Fig. 1), which precluded the use of 21-day cultures as a model of endothelial monolayer barrier function. Day 11 cultures had no basement membrane, but importantly overlapping of cells was not seen.

3.2. Human albumin ELISA

Albumin is a major determinant of plasma osmotic pressure. Increased endothelial permeability to albumin in response to inflammatory mediators is a crucial pathological event in syndromes characterized by vascular leak, including dengue haemorrhagic fever. Transendothelial transport of albumin has therefore been studied extensively in animals, isolated-perfused whole vessel preparations and, more recently, tissue culture models (reviewed in Michel, 1996). There is increasing appreciation of the complexity of transendothelial albumin transport, raising the concern that artificial tracers including labelled albumin may behave differently from native albumin. This is particularly likely if large molecules such as FITC or trypan blue are used for labelling and may even occur as a result of the process of radiolabelling (Freeman, 1959). Therefore, a reliable method for detection of native human albumin in serum by ELISA was established. Fig. 2 shows a typical standard curve using HS containing a known albumin concentration, which demonstrates good discrimination of albumin concentration within a working range of 1–100 μg/ml. The overall coefficient of variation over many repeat analyses of the same samples within this range was calculated to be 8.5–10% (data not shown).

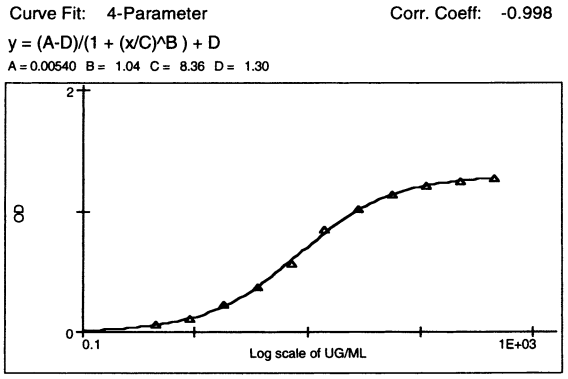

Fig. 2. Example standard curve in albumin ELISA. A HS sample containing a known concentration of albumin was serially diluted in RPMI 1640 in duplicate and analysed by ELISA. The plate was read in an automated plate reader and the standard curve calculated using plate reader software (Molecular Devices).

3.3. Albumin permeability of unstimulated monolayers

Several different calculated values have been used to quantitate the permeability of endothelium to macromolecules such as albumin. In many cases, these values (such as percent transfer (Brett et al., 1989) and clearance (Goldblum et al., 1994)) are applicable only within the particular study and do not permit comparison with other studies using different methods, even if the experimental protocols are broadly similar. In contrast, calculation of PC corrects for the concentration gradient across the monolayer, area of the membrane, length of experiment and chamber volumes (Casnocha et al., 1989). Furthermore, directly comparable values of PC have been calculated from in vivo studies (reviewed in Michel, 1996). Some different HUVEC culture conditions were tested to determine their effect on PC for albumin. The larger the value (in cm/s) of the PC for albumin, the more permeable the endothelial barrier to albumin.

3.4. Fetal calf vs. human serum

HUVECs are highly dependent on serum for growth in tissue culture. FCS has been used almost universally as a source of serum for HUVEC

culture, including all previously reported endothelial permeability models to our knowledge. In this study, the effect of substituting HS for FCS on HUVEC barrier function was examined. HUVECs from the same primary cultures were subcultured in cell culture medium containing either 20% FCS or 20% HS, and then transendothelial albumin flux was measured at day 11. It was consistently found that the HUVEC monolayers grown in HS had significantly lower PCs for albumin when compared with the same cells grown in FCS (Fig. 3). The simple modification of using HS to grow HUVECs from the first subculture was therefore adopted in all subsequent experiments.

3.5. Time-course of cultured HUVEC permeability

Beynon et al. (1993) found that the permeability of their HUVEC monolayer model was lowest at 4–5 days in culture, and thereafter increased with more prolonged culture. By contrast, Casnocha et al. (1989) demonstrated that the PC for albumin diminished progressively up to 15 days in culture, although the difference between days 11 and 15 was small. These studies serve to emphasize that it was difficult to infer the optimal

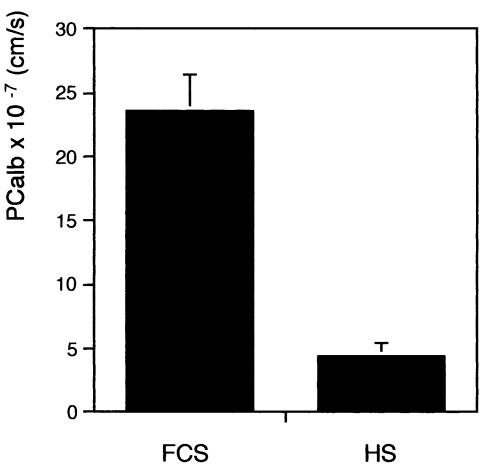

Fig. 3. Comparison of barrier function of HUVEC monolayer cultured in FCS or HS. Each PC value is the mean of triplicate experiments. The error bars show 1 standard error of the mean.

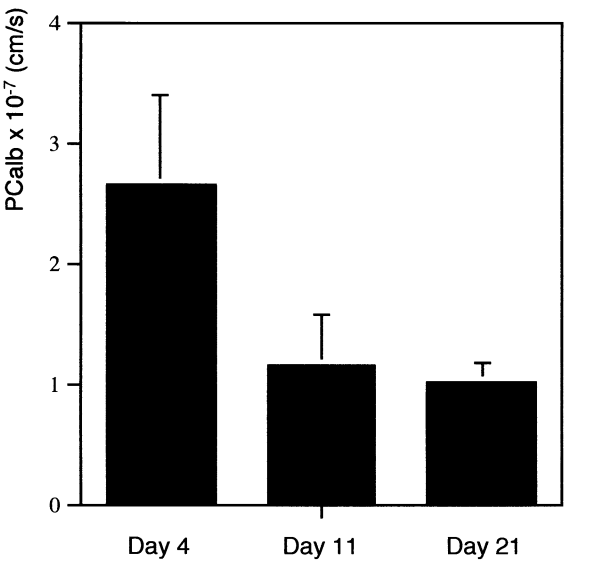

Fig. 4. Time-course of cultured HUVEC barrier. Time in culture is shown on the x-axis. Each PC value is the mean from three separate time-course experiments, each performed in triplicate. The error bars show 1 standard error of the mean.

conditions to generate a HUVEC monolayer permeability model from the existing literature, due to the heterogeneity of techniques described. The effect of time in culture was tested in the model by measuring transendothelial albumin flux across HUVEC monolayers grown from the same donor at days 4, 11 and 21. Unlike previous studies, the functional results could be correlated with EM morphological data. There was a consistent improvement in barrier function with longer cultures (Fig. 4). The difference in permeability after 21 days in culture compared with 11 days was small. Given the morphological data showing that the cell monolayer best reflected in vivo characteristics at day 11 (see above), this time point was selected for all subsequent experiments.

3.6. Molecular sieving

There are conflicting data regarding the ability of cultured endothelial cell monolayers to sieve macromolecules (reviewed in Turner, 1992). In order to determine whether or not the HUVEC monolayer barrier was capable of sieving macro-

molecules, its permeability to albumin and urea were compared. The rate of diffusion (or free diffusion coefficient, D) of molecules is related inversely to molecular size, which means that even in the absence of any selectivity the mass flux across a barrier over time of a large molecule, such as albumin ($D = 5.94 \times 10^{-7}$ cm^2/s), is lower than the mass flux of a small molecule, such as urea ($D = 1.1 \times 10^{-5}$ cm^2/s). Therefore, direct comparison of PCs of large and small molecules does not enable demonstration of molecular sieving. Instead, PC/D was calculated for urea and albumin to assess selectivity of the HUVEC monolayer to molecules of different size (Siflinger-Birnboim et al., 1987). If there is free diffusion across a barrier then PC/D does not vary with molecular size, whereas if larger molecules are restricted to a greater extent than smaller molecules then PC/D decreases with increasing molecular size. This calculation makes the assumption that there is no transport of macromolecules by convection, which may be a particular issue with regard to cell-free membranes (Katz and Schaeffer, 1991) which are used to calculate the PC of the endothelial monolayer without the contribution of the membrane (Casnocha et al., 1989). However, Siflinger-Birnboim et al. (1987) showed that, as expected, there is no molecular sieving by cell-free, gelatinized membranes. Therefore, total PC (cell monolayer and coated membrane)/D was used to assess molecular sieving in the model and it was assumed that if there were evidence of restricted diffusion then this could be attributed to the cell monolayer rather than the membrane.

Table 1
Molecular sieving by cultured HUVEC monolayers

	Molecular radius (nm)	PC/D (per cm)	1 SEM
Urea	0.22	10.2	0.09
Albumin	3.5	0.23	0.01

Mass flux of urea and albumin across HUVEC monolayers was measured in triplicate experiments, and PC divided by free diffusion coefficient (D) was calculated for each molecule. SEM, standard error of mean.

Table 1 shows PC/D for urea and albumin. There was a striking inverse correlation of PC/D with molecular size, at least for these two molecules. This implies that the endothelial barrier was selective with regard to molecular size, in a manner resembling endothelium in vivo.

3.7. Effect of TNF-α on endothelial permeability

TNF-α is a pro-inflammatory cytokine that has been implicated widely in conditions associated with vascular leak, including dengue haemorrhagic fever (Anderson et al., 1997; Bethell et al., 1998; Green et al., 1999), and has been shown previously to increase permeability of cultured endothelial monolayers (Brett et al., 1989; Beynon et al., 1993; Burke-Gaffney and Keenan, 1993). Having developed the HUVEC monolayer model of endothelial barrier function, it was then exposed to rTNF-α as a model agonist and the effect of rTNF-α on albumin permeability was measured. Results are expressed as $\log_{10}$-transformed ratios of exposed to unexposed PCs for albumin. If rTNF-α has no effect on permeability, then this value is 0; increased permeability of exposed cells results in positive values, decreased permeability in negative values.

The results of 24 h exposure to rTNF-α are shown in Fig. 5. In keeping with previous data, TNF-α increased endothelial permeability. In this model, 40 U/ml rTNF-α induced significant leak ($P = 0.008$), and a dose-dependent increase in endothelial monolayer permeability was observed. The effect of rTNF-α on permeability was abrogated by heat-inactivation, which excluded an effect of contaminating endotoxin, which is heat-stable.

In order to demonstrate that rTNF-α caused a reversible functional change in the cultured HUVEC monolayer, some cell monolayers were allowed to recover for 24 h following exposure to TNF-α before repeating the measurement of transendothelial albumin flux. Unexpectedly, it was found that endothelial monolayers that had been exposed previously to low-dose rTNF-α (40 U/ml) had reduced significantly permeability when compared with unexposed monolayers ($P = 0.002$) (Fig. 6). This was not examined further in

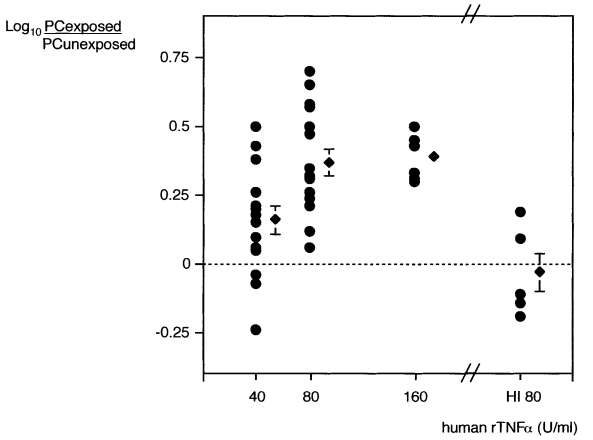

Fig. 5. TNF-α increases permeability of HUVEC monolayers. Cultured HUVEC monolayers were exposed to human recombinant TNF-α (rTNF-α) at various doses for 24 h. In some experiments, rTNF-α was first HI. Albumin permeability of exposed and unexposed monolayers was then measured, and the log-transformed ratio of PC calculated as described in the text. Mean values (◆) ± 1 SEM are shown. A value of 0 indicates no change in permeability in exposed monolayers.

this study. At the single recovery time-point used, the permeability of monolayers exposed previously to a higher dose of rTNF-α (80 U/ml) did not differ significantly from unexposed monolayers ($P = 0.45$), that is they had recovered to baseline (Fig. 6).

4. Discussion

Vascular leak is a key pathophysiological feature in dengue haemorrhagic fever, yet the underlying mechanisms of endothelial injury are not understood. Studies have been hampered by the lack of an animal model of dengue haemorrhagic fever. In order to advance the study of endothelial injury in dengue haemorrhagic fever, an in vitro model of the endothelial barrier was developed. Similar models have been reported widely, but there is no consensus regarding the best method. This model incorporates several important improvements that enhance its applicability for studies of vascular leak in human diseases such as dengue haemorrhagic fever.

Some empirical choices were made regarding the structure of this model based on the existing literature. The model was based on culture of primary HUVECs, despite the fact that this is a relatively demanding technique. There are limited alternative sources of primary human endothelial cells, and others (such as placenta) yield a mixed population of microvascular endothelial cells which is not ideal given the heterogeneity of endothelium at different sites (reviewed in Cines et al., 1998). Although human endothelial cell lines have been established and are convenient for some studies, many lack important characteristics for development of an endothelial monolayer barrier, in particular contact inhibition of growth which stops cells from piling up into multiple layers. All experiments were carried out with first subculture HUVECs because HUVEC morphology and function may change with increasing passage number. Membranes with small pores were used to prevent endothelial cell growth into the pores (Albelda et al., 1988).

Similar models have been reported but their physiological relevance has been questioned (Al-

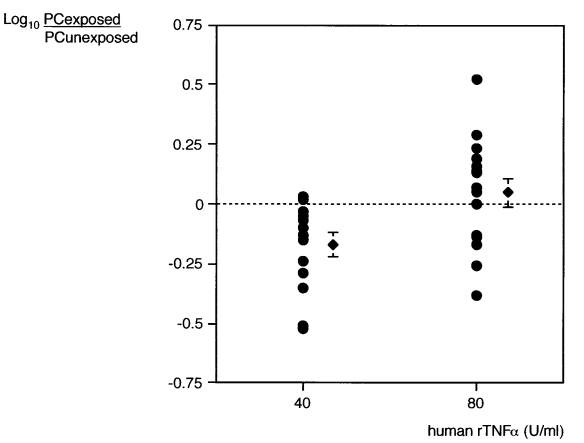

Fig. 6. HUVEC monolayer permeability recovers after exposure to TNF-α. Cultured HUVEC monolayers were exposed to human recombinant TNF-α (rTNF-α) at various doses for 24 h. TNF-α was then removed, and albumin permeability of previously exposed and unexposed monolayers was measured after 24 h recovery. The log-transformed ratio of PC were calculated as described in the text. Mean values (◆) ± 1 SEM are shown. A value of 0 indicates no change in permeability in exposed monolayers.

belda et al., 1988; Michel, 1996). One particular criticism is that monolayers of cultured endothelial cells are usually 100 times more permeable to macromolecules such as albumin than intact microvascular endothelium. The reasons for this discrepancy are unclear. The model reported here was characterized both morphologically and functionally in order to optimize these parameters in comparison with microvascular endothelium.

The cross-sectional morphology of cultured HUVECs at day 11, as judged by EM, was similar to the morphology of microvascular endothelial cells in tissue sections. Importantly, EM demonstrated that day 11 HUVEC cultures grew in a continuous monolayer without overlapping of cells. The surface glycocalyx, which may play an important role in molecular sieving (Curry and Michel, 1980), was visible after staining with alcian blue on the luminal surface of the endothelial cell monolayer. One striking morphological difference between the cultured HUVEC monolayer and microvascular endothelium in vivo was the absence of a basement membrane. Although the basement membrane makes a relatively small contribution to the normal permeability characteristics of the vessel wall in a passive sense when compared with the glycocalyx and cell monolayer, it exerts an important influence on endothelial cell growth and adhesion. Production of a basement membrane is therefore a desirable characteristic of in vitro vessel wall constructs. The likely absence of a basement membrane has been generally overlooked in previous reports of in vitro endothelial barrier models.

Baseline PCs for albumin for the endothelial monolayer in the range $1–4 \times 10^{-7}$ cm/s were achieved, which is approximately 10 times lower than for previously reported cultured endothelial barriers (Albelda et al., 1988; Casnocha et al., 1989; Michel, 1996). Much of this improvement in barrier function can be attributed to growing HUVECs in HS rather than FCS, which has been used previously. Testing whether or not substituting HS for FCS influenced barrier function was a logical step given the human origin of endothelial cells in this model. It was reasoned that homologous serum would contain many components that take part in the complex interaction between plasma and the endothelial cell glycocalyx, which influences vascular permeability (Adamson and Clough, 1991; Huxley and Curry, 1991). Moreover, angiogenesis is a complex process (reviewed in Cines et al., 1998) and soluble factors in HS might alter the growth characteristics of the HUVEC monolayer. The fact that primary culture of HUVECs in HS was unsuccessful due to overgrowth of fibroblasts served to emphasize that there are important differences between HS and FCS with respect to endothelial cell growth.

TNF-α induced a reversible increase in permeability in a dose-responsive manner. This supported the conclusion that the cultured endothelial monolayer had functional characteristics that resembled endothelial cells in vivo and was well suited to further studies relating to the pathogenesis of dengue haemorrhagic fever. Increased permeability was observed at lower doses of TNF-α than generally reported using cultured human endothelial monolayers, although valid comparison can only be made with studies which express the TNF-α dose in terms of biological activity (U/ml). Comparison with the previous endothelial barrier model developed specifically for the study of dengue haemorrhagic fever is particularly relevant. Bonner and O'Sullivan (1998) developed a tissue culture model of permeability based on transendothelial flux of trypan-blue labelled albumin across a cultured human endothelial cell line, ECV304. This model appears to lack many of the desirable characteristics for an in vitro model of the endothelial barrier for studies of dengue haemorrhagic fever. Most importantly, barrier function of the cultured cell line does not seem to resemble closely that of in vivo endothelium, although direct comparison is difficult because a non-physiological tracer was used and results are presented as clearance rather than PC. Large doses (500–1000 U/ml) of TNF-α were required to induce an increase in permeability and it was not possible to demonstrate a dose–response to TNF-α. Also, cultures became unresponsive to TNF-α with increasing time in culture, but the possibility that the cell line was piling up was not considered and no morphological data was included in the report.

Significant variation in baseline permeability of unstimulated cells grown from different donors was observed. Functional variation amongst genetically distinct HUVECs is well established (Bender et al., 1994). In most experiments, baseline PC for albumin was in the range $1–4 \times 10^{-7}$ cm/s. However, in some experiments baseline PC for albumin was higher. The reasons for this variation were not examined. Increased baseline permeability, especially if it were caused by unrecognized gaps in the monolayer, would be expected to mask small changes in permeability. Therefore, once the model had been established, it was decided to exclude prospectively each experiment in which baseline PC for albumin was found to be greater than 4×10^{-7} cm/s. Subsequently it was found that nearly one in four cultures were excluded for this reason. This represents a significant disadvantage for this model system because the baseline PC for albumin could only be calculated at the end of each experiment, by which stage considerable time and resources would have been committed to an experiment that might have to be excluded.

Despite the advances made in this cultured endothelial cell monolayer in comparison with previous models, it remains an approximation of the vessel wall in vivo. Permeability coefficients for albumin in the range $1–4 \times 10^{-7}$ cm/s are still approximately 10 times more permeable than estimates of microvascular permeability in vivo (Michel, 1996). This may be a consequence of failure of the cultured endothelial monolayer to reflect accurately the endothelial layer in intact vessels. Activation of endothelial cells simply by virtue of tissue culture (Cines et al., 1998) might impair their barrier function. Albelda et al. (1988) attributed the leakiness of their culture monolayer to small gaps between a small proportion of cultured endothelial cells. Although no gaps were observed in the monolayer by EM, it was possible that these were missed due to sampling error. Furthermore, the cultured monolayer lacked exposure to hydrostatic pressure, which has been implicated in remodelling of endothelium resulting in reduced permeability (Suttorp et al., 1988). Alternatively, Casnocha et al. (1989) argued that cultured endothelial monolayer models might better reflect the endothelial permeability barrier function in vivo than PC measurements indicate. Other vessel wall components that are lacking in tissue culture models, such as smooth muscle, may contribute to the vascular permeability barrier in vivo.

The accuracy of measurement of PCs also requires further scrutiny. The method makes the assumption that there is no change in volume of the luminal and subluminal compartments over the time course of the measurement of transendothelial albumin flux. However, a corollary of the finding that the HUVEC monolayer was sieving macromolecules is that the presence of 40% HS in the luminal compartment must exert an osmotic pressure. A net flux of water and small solutes would therefore be expected from the subluminal to luminal compartment, which may have reduced the accuracy of measurement of permeability coeffient. A further refinement to the method, although technically difficult, would be the application of hydrostatic pressure on the luminal side of the monolayer, to mimic the balance of hydrostatic and osmotic forces in vivo.

The unstirred layer phenomenon has been mostly ignored in the use of cultured endothelial monolayers for permeability measurements. Unstirred layers of fluid apposed to each side of the membrane represent a permeability barrier equal to D/δ, that is the free diffusion coefficient of a molecule divided by the thickness of the unstirred layer (δ) (Barry and Diamond, 1984). Siflinger-Birnboim et al. (1987) argued that the unstirred layer sizes are likely to be similar with and without the endothelial monolayer. Therefore, the contribution of the unstirred layers to the endothelial permeability barrier is potentially eliminated in the calculations when the contribution of the coated membrane is subtracted. This assumption is not valid if there is significant convective flux across membranes without cells (Barry and Diamond, 1984; Katz and Schaeffer, 1991), in which case a component of the calculated PC may be attributable to the unstirred layer phenomenon. Vigorous stirring was used in order to minimize this effect, but the magnitude of this phenomenon in this model is unknown.

The cultured endothelial cell monolayer model reported here represents a significant advance in comparison with previous studies and provides the opportunity to extend in vitro studies on the pathogenesis of vascular leak in dengue haemorrhagic fever. Although caution would need to be exercised when extrapolating from this model to human disease, it may be possible to obtain important clues regarding the mechanisms underlying vascular leak in dengue haemorrhagic fever.

Acknowledgements

We thank C. Neal for performing electron microscopy and Professor C. Michel for helpful discussions. M.J. is funded by The Wellcome Trust.

References

Albelda, S.M., Sampson, P.M., Haselton, F.R., McNiff, J.M., Mueller, S.N., Williams, S.K., Fishman, A.P., Levine, E.M., 1988. Permeability characteristics of cultured endothelial cell monolayers. J. Appl. Physiol. 64, 308–322.

Adamson, R.H., Clough, G., 1991. Plasma proteins modify the endothelial cell glycocalyx of frog mesenteric microvessels. J. Physiol. 445, 473–486.

Anderson, R., Wang, S., Osiowy, C., Issekutz, A.C., 1997. Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. J. Virol. 71, 4226–4232.

Barry, P.H., Diamond, J.M., 1984. Effects of unstirred layers on membrane phenomena. Physiol. Rev. 64, 763–872.

Bender, J.R., Sadeghi, M.M., Watson, C., Pfau, S., Pardi, R., 1994. Heterogeneous activation thresholds to cytokines in genetically distinct endothelial cells: evidence for diverse transcriptional responses. Proc. Natl. Acad. Sci. USA 91, 3994–3998.

Bethell, D.B., Flobbe, K., Phuong, C.X.T., Day, N.P.J., Phuong, P.T., Buurman, W.A., Cardosa, M.J., White, N.J., Kwiatkowski, D., 1998. Pathophysiologic and prognostic role of cytokines in dengue haemorrhagic fever. J. Infect. Dis. 177, 778–782.

Beynon, H.L.C., Haskard, D.O., Davies, K.A., Haroutunian, R., Walport, M.J., 1993. Combinations of low concentrations of cytokines and acute agonists synergize in increasing the permeability of endothelail monolayers. Clin. Exp. Immunol. 91, 314–319.

Bonner, S.M., O'Sullivan, M.A., 1998. Endothelial cell monolayers as a model system to investigate dengue shock syndrome. J. Virol. Methods 71, 159–167.

Brett, J., Gerlach, H., Nawroth, P., Steinberg, S., Godman, G., Stern, D., 1989. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J. Exp. Med. 169, 1977–1991.

Burke-Gaffney, A., Keenan, A.K., 1993. Modulation of human endothelial cell permeability by combinations of the cytokines interleukin-1 alpha/beta, tumor necrosis factor-alpha and interferon gamma. Immunopharmacology 25, 1–9.

Casnocha, S.A., Eskin, S.G., Hall, E.R., McIntire, L.V., 1989. Permeability of human endothelial monolayers: effects of vasoactive agonists and cAMP. J. Appl. Physiol. 67, 1997–2005.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, B.S., McCrae, K.R., Hug, B.A., Schmidt, A.M., Stern, D.M., 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 91, 3527–3561.

Curry, F.E., Michel, C.C., 1980. A fiber matrix model of capillary permeability. Microvasc. Res. 20, 96–99.

Freeman, T., 1959. The biological behaviour of normal and denatured human plasma albumin. Clin. Chim. Acta 4, 788–792.

Goldblum, S.E., Brann, T.W., Ding, X., Pugin, J., Tobias, P.S., 1994. Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function, in vitro. J. Clin. Invest. 93, 692–702.

Green, S., Vaughn, D.W., Kalayanarooj, S., Nimmannitya, S., Suntayakorn, S., Nisalak, A., Lew, R., Innis, B.L., Kurane, I., Rothman, A., Ennis, F.A., 1999. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J. Infect. Dis. 179, 755–762.

Halstead, S.B., 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239, 476–481.

Halstead, S.B., 1989. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev. Infect. Dis. 11, S830–S839.

Huber, A.R., Weiss, S.J., 1989. Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. J. Clin. Invest. 83, 1122–1136.

Huxley, V.H., Curry, F.E., 1991. Differential actions of albumin and plasma on capillary solute permeability. Am. J. Physiol. 260, H1645–H1654.

Jaffe, E.A., Nachman, N.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest. 52, 2745–2764.

Katz, M.A., Schaeffer, R.C.J., 1991. Convection of macromolecules is the dominant mode of transport across horizontal 0.4- and 3-micron filters in diffusion chambers: significance for biologic monolayer permeability assessment. Microvasc. Res. 41, 149–163.

Michel, C.C., 1996. Transport of macromolecules through microvascular walls. Cardiovasc. Res. 32, 644–653.

Michel, C.C., 1998. Capillaries, caveolae, calcium and cyclic nucleotides: a new look at microvascular permeability. J. Mol. Cell. Cardiol. 30, 2541–2546.

Ramirez, C.A., Colton, C.K., Smith, K.A., Stemerman, M.B., Lees, R.S., 1984. Transport of 125I-albumin across normal and deendothelialized rabbit thoracic aorta in vivo. Arteriosclerosis 4, 283–291.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., de Mesa, M.T., Nogueira, R.M., da Rosa, A.T., 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230, 244–251.

Siflinger-Birnboim, A., Del Vecchio, P.J., Cooper, J.A., Blumenstock, F.A., Shepard, J.M., Malik, A.B., 1987. Molecular sieving characteristics of the cultured endothelial monolayer. J. Cell Physiol. 132, 111–117.

Simionescu, M., Simionescu, N., 1984. Ultrastructure of the microvascular wall: functional correlations. In: Renkin, E.M., Michel, C.C. (Eds.), Handbook of Physiology. Williams and Wilkins Co, Baltimore, pp. 41–101.

Suttorp, N., Hessz, T., Seeger, W., Wilke, A., Koob, R., Lutz, F., Drenckhahn, D., 1988. Bacterial exotoxins and endothelial permeability for water and albumin in vitro. Am. J. Physiol. 255, C368–C376.

Turner, M.R., 1992. Effects of proteins on the permeability of monolayers of cultured bovine arterial endothelium. J. Physiol. 449, 21–35.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., Nisalak, A., 2000. Dengue viremia titre, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. 181, 2–9.

Watts, D.M., Porter, K.R., Putvatana, P., Vasquez, B., Calampa, C., Hayes, C.G., Halstead, S.G., 1999. Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet 354, 1431–1434.