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Filovirus-Induced Endothelial Leakage Triggered by Infected Monocytes/Macrophages

HEINZ FELDMANN,[1] HARALD BUGANY,[1] FRIEDERIKE MAHNER,[1] HANS-DIETER KLENK,[1] DETLEY DRENCKHAHN,[2] AND HANS-JOACHIM SCHNITTLER[2]*

Institut für Virologie, Philipps-Universität Marburg, D-35037 Marburg,[1] and Anatomisches Institut, Julius-Maximilians Universität Würzburg, D-97070 Würzburg,[2] Germany

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The pathogenetic mechanisms underlying viral hemorrhagic fevers are not fully understood, but hemorrhage, activation of coagulation, and shock suggest vascular instability. Here, we demonstrate that Marburg virus (MBG), a filovirus causing a severe form of hemorrhagic fever in humans, replicates in human monocytes/macrophages, resulting in cytolytic infection and release of infectious virus particles. Replication also led to intracellular budding and accumulation of viral particles in vacuoles, thus providing a mechanism by which the virus may escape immune surveillance. Monocytes/macrophages were activated by MBG infection as indicated by tumor necrosis factor alpha (TNF-α) release. Supernatants of monocyte/macrophage cultures infected with MBG increased the permeability of cultured human endothelial cell monolayers. The increase in endothelial permeability correlated with the time course of TNF-α release and was inhibited by a TNF-α-specific monoclonal antibody. Furthermore, recombinant TNF-α added at concentrations present in supernatants of virus-infected macrophage cultures increased endothelial permeability in the presence of 10 μM H_2O_2. These results indicate that TNF-α plays a critical role in mediating increased permeability, which was identified as a paraendothelial route shown by formation of interendothelial gaps. The combination of viral replication in endothelial cells (H.-J. Schnittler, F. Mahner, D. Drenckhahn, H.-D. Klenk, and H. Feldmann, J. Clin. Invest. 19:1301–1309, 1993) and monocytes/macrophages and the permeability-increasing effect of virus-induced cytokine release provide the first experimental data for a novel concept in the pathogenesis of viral hemorrhagic fever.

Viral hemorrhagic fevers (HFs) are caused by members of the *Arenaviridae*, *Bunyaviridae*, *Flaviviridae*, and *Filoviridae* families (33). Important examples of viral HFs are (i) HF with renal syndrome, an emerging health problem in Asia, Europe, and, recently, the United States (24, 30); (ii) Lassa fever, a major cause of severe disease in Africa (1); and (iii) viral HF caused by New World arenaviruses in South America (27). Filoviruses cause the most severe forms of viral HF that are associated with multiple hemorrhagic manifestations, marked hepatic involvement, disseminated intravascular coagulation (DIC), shock, and the highest case-fatality rates (30 to 90%) (12, 28, 33). Filoviruses are enveloped, nonsegmented, negative-strand RNA viruses and constitute a separate family within the order *Mononegavirales* (9, 20). The genomes and the structural proteins of Marburg virus (MBG) and Ebola virus have been studied extensively during the past few years (9, 10, 36). The general principles of viral replication have also been unraveled, but little is known about the pathogenesis of filoviral HF. Virus-induced dysfunction of the endothelium, including endothelial cell damage and increased permeability, has been thought to be a factor responsible for some of the major clinical symptoms observed in viral HF patients (33, 34). Endothelial cells control the exchange of macromolecules and water, regulate the vascular tone by release of autacoids, and are involved in the control of the coagulation and anticoagulation cascades (8, 25, 26, 38). Recently it was demonstrated that filoviruses replicate in cultured human endothelial cells (39) and endothelial cells of infected monkeys (14). Replication in endothelial cells in vitro resulted in the production of infectious viral particles. Infection of endothelial cells has been observed in vivo (14), and endothelial cells infected in organ culture show cell lysis (39). It is therefore reasonable to assume that endothelial cell damage contributes to the gross bleeding tendency during filovirus infection. However, the fulminant shock symptom associated with generalized edematous swelling seen in later stages of the disease (28, 33) suggests that there are additional factors contributing to increased endothelial permeability and disturbed hemostasis.

Recent morphologic studies of filovirus-infected monkeys suggested viral replication in circulating monocytes/macrophages and tissue macrophages (14). Monocytes/macrophages are part of the mononuclear phagocyte system, which is involved in immunologic and inflammatory responses. The release of humoral factors after stimulation is an important mechanism by which monocytes/macrophages attack their targets and cause inflammatory responses. In fact, the prototype cytokine tumor necrosis factor alpha (TNF-α) has been shown to mediate the development of shock symptomatic in septic patients. Therefore, we examined the effect of virus-induced activation of monocytes/macrophages on endothelial permeability in vitro. MBG, the prototype filovirus, was chosen as a model for these studies. In this paper, we demonstrate that MBG infection of monocytes/macrophages stimulates TNF-α release, which in turn leads to an increase in paraendothelial permeability. Furthermore, replication of MBG in monocytes/macrophages resulted in the release of infectious viral particles and finally caused cell lysis.

MATERIALS AND METHODS

Virus. All infections were performed with the Musoke strain of MBG isolated in 1980 in Kenya (41).

* Corresponding author. Mailing address: Department of Anatomy, Julius-Maximilians Universität Würzburg, Koellikerstr. 6, D-97070 Würzburg, Germany. Phone: 49 931-312706. Fax: 49 931-15988.

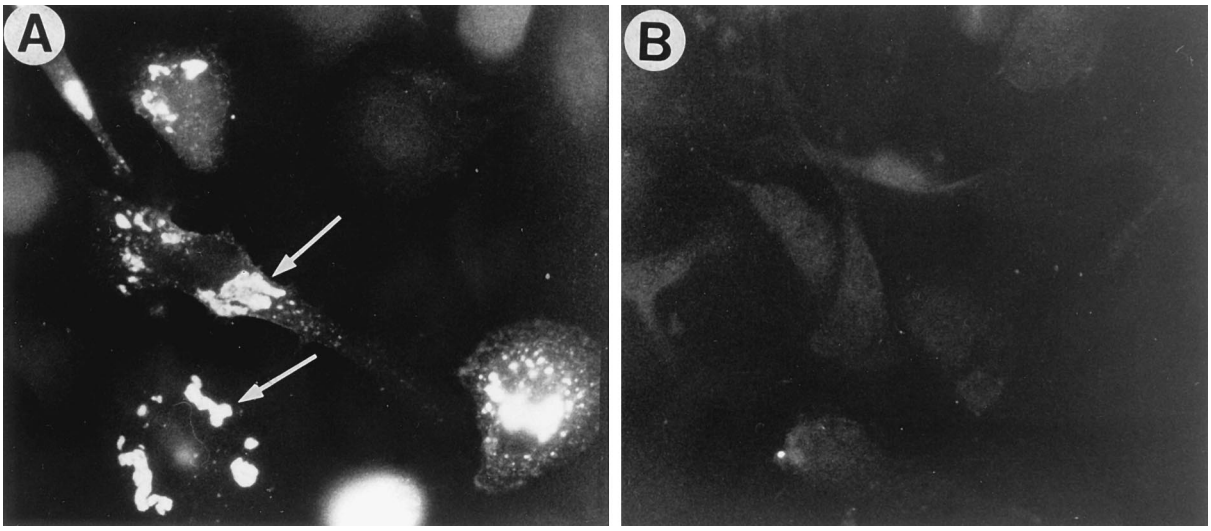

FIG. 1. Primary cultures of human monocytes/macrophages stained with an MBG (Musoke)-specific human reconvalescent-phase serum 9 days p.i. (A) Viral inclusion bodies were observed in MBG-infected monocytes/macrophages (arrows). (B) Mock-infected cells showed only background staining.

Culturing of monocytes/macrophages from human blood. Peripheral blood mononuclear cells were prepared from buffy coats of healthy donors from the local blood bank. The mononuclear fraction was separated by standard methods (19). Cells were finally resuspended in RPMI 1640 supplemented with 5% heat-inactivated pooled human serum (Gibco, Eggenstein, Germany). To separate adherent monocytes/macrophages from nonadherent cells, 5×10^6 cells were plated in 24-well tissue culture plates, incubated for 1 h at 37°C, and subsequently washed to remove nonadherent cells. Adherent cells were cultured for 7 days in RPMI 1640 containing 5% human serum in order to obtain a macrophage-like morphology (19). In addition, cells were identified as monocytes/macrophages by indirect immunofluorescence with a monoclonal antibody directed to CD14 (Dako, Hamburg, Germany).

Human endothelial cell culture. Human umbilical vein endothelial cells (HUVECs) were collected and cultured as described previously (39, 40).

Plaque assay. Confluent Vero E6 cell monolayers cultured in six-well tissue culture plates were infected with clarified tissue culture supernatant of MBG-infected Vero E6 cells, HUVECs, and monocytes/macrophages at dilutions ranging from 10^{-1} to 10^{-8} (39). Culturing of Vero E6 cells (CRL 1586; American Type Culture Collection, Rockville, Md.) and infection of Vero E6 cells and HUVECs (multiplicity of infection [MOI] of 10^{-2} PFU per cell), used for controls, were performed as described previously (11, 39).

In vivo labeling and analysis of viral proteins. Labeling was performed at day 5 postinfection. Infected cells were washed once with methionine-free RPMI (Gibco, Eggenstein, Germany) and incubated at 37°C for 24 h with methionine-free medium containing 20 μCi of [^{35}S]methionine (Amersham Buchler, Braunschweig, Germany) per ml. Labeled viral particles were purified, and structural proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [10% polyacrylamide]) (15).

Mode of infection. Monocytes/macrophages were infected with MBG Musoke at either an MOI of 10 (activation studies) or at 10^{-2} PFU per cell (replication studies) 7 days after cultivation. Adsorption of viral particles was performed for 1 h at 37°C. Subsequently, the inoculum was removed, and the cells were washed twice with phosphate-buffered saline (PBS). RPMI supplemented with 5% human pooled serum from healthy donors was added, and incubation proceeded for the appropriate time at 37°C.

Immunofluorescence. Monocytes/macrophages were fixed with 2% formaldehyde in PBS for 10 min, washed twice with PBS, permeabilized in acetone (−20°C) for 1 min, and processed for indirect immunofluorescence microscopy as described previously (39). Viral proteins were detected with either an MBG (Musoke)-specific human reconvalescent-phase serum or a monospecific anti-MBG (Musoke) nucleoprotein serum (guinea pig) (2) followed by the corresponding secondary antibody labeled with fluorescein isothiocyanate (Sigma, Deisenhofen, Germany). Endothelial cells were fixed with methanol (−20°C, 10 min), washed in PBS, and further subjected to indirect immunofluorescence with a monoclonal antibody directed to plakoglobin (Progen, Heidelberg, Germany).

Transmission electron microscopy. For transmission electron microscopy, monocytes/macrophages were cultured on polycarbonate filters (Costar, Cambridge, Mass.) and infected as described above. Filters were fixed in 2% glutaraldehyde (Sigma) and processed as described previously (39). Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were observed and photographed with a Zeiss E 109 electron microscope.

Transwell filter system (permeability assay). Permeability assays were performed with a modified version of a previously published system (22). Polycarbonate filters (3-μm-pore size and 6-mm diameter [Costar]) were coated with 0.1% gelatin for 2 h. Subsequently, the gelatin was cross-linked for 30 min with 2% glutaraldehyde (in PBS). After an excessive wash with PBS overnight, filters were put into 24-well plates and HUVECs were seeded on the coated filter membranes (5×10^4 cells per cm^2) in 300 μl of medium 199 (upper compartment) and cultured until confluence (2 days). The lower compartments contained 700 μl of medium 199. Prior to the permeability assay, HUVEC monolayers (filter units) were washed twice with RPMI 1640 and transferred to a new 24-well plate. The monolayers were incubated at 37°C with 300 μl of filtered tissue culture fluids (0.1-μm-pore size) added to the upper compartment, followed by 0.25 mM horseradish peroxidase (HRP) 30 min later. Aliquots of 60 μl were collected from the lower compartment at different time intervals and stored immediately at −20°C. HRP activity was determined by addition of 860 μl of reaction buffer (5 mM guaiacol in 50 mM NaH_2PO_4), and 100 μl of H_2O_2 (0.6 mM in H_2O, freshly prepared). After an incubation period of 25 min at room temperature, A_{470} was measured. For the inhibition studies, a monoclonal antibody directed to TNF-α (clone 195; Boehringer, Mannheim, Germany) was added at 60 μg/ml (an amount that neutralizes about 30 ng of recombinant TNF-α per ml) to the culture supernatants of MBG-infected monocytes/macrophages. After an incubation time of 30 min at 37°C, the supernatants were placed in the upper compartment of the transwell filter system and assayed for permeability induction as described above. For further control experiments, different concentrations of recombinant TNF-α in the presence or absence of H_2O_2 were tested for their ability to increase endothelial permeability. Monolayers were treated for 6 h with recombinant TNF-α at 37°C prior to addition of H_2O_2 (10 μM) and HRP.

TNF-α ELISA. Monocytes/macrophages cultured in six-well plates were infected with MBG Musoke at an MOI of 10 PFU per cell. One milliliter of tissue culture supernatant was collected at different times postinfection and stored at −20°C. Determination of TNF-α in the culture supernatants was performed with an enzyme-linked immunosorbent assay (ELISA) that specifically reacts with human TNF-α (Boehringer) according to the manual of the manufacturer.

Reverse transcriptase PCR. Viral RNA isolation, reverse transcriptase PCR, and hybridization were performed as described recently (39) with the following oligonucleotides: 2232(+), ACTTTTATAGCCCACCACATTGTGTGA; and 2449(−), TCATAGAGTTGTTATACATTGATTATC (L gene of MBG Musoke [EMBL Data Library, emnew: MVREPCYC, accession no. Z12132]).

RESULTS

Replication of MBG in human monocytes/macrophages. Monocytes/macrophages were susceptible to infection with MBG and produced infectious viral particles. Two days postinfection (p.i.), cells displayed the first signs of virus antigen production, as revealed by immunofluorescence. Within a period of 9 to 12 days p.i., most cells were positive for MBG antigen. The immunoreaction displayed punctate and plaque-like patterns, which are known as inclusion bodies and which

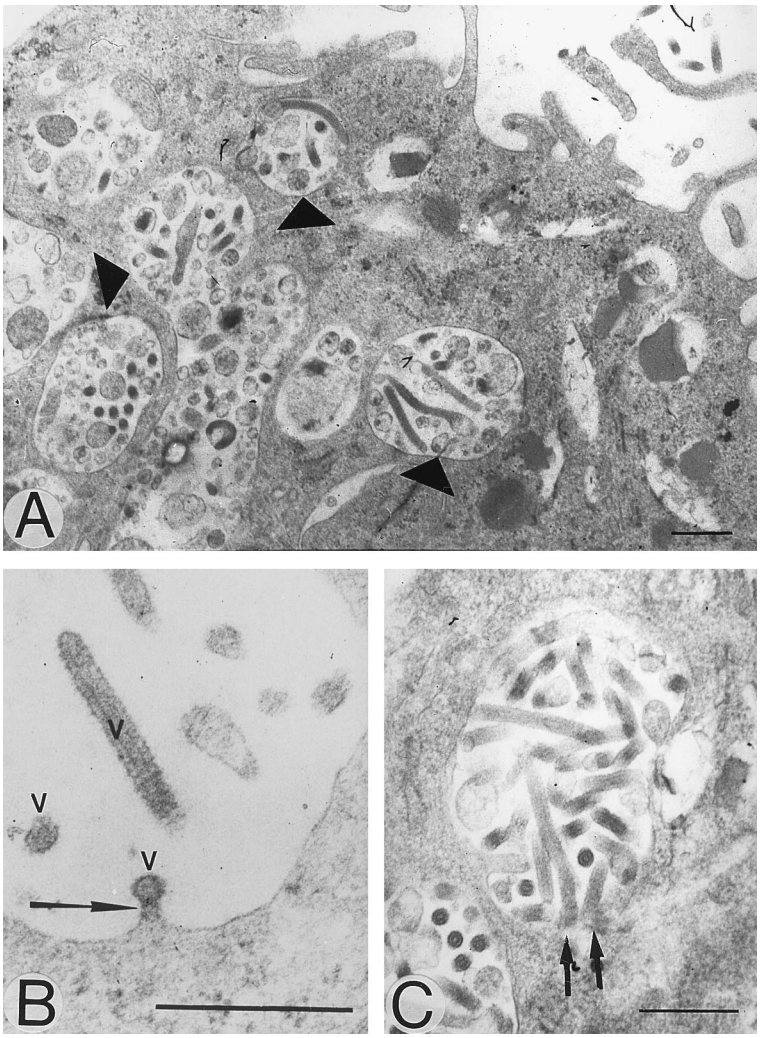

FIG. 2. Transmission electron microscopy of primary cultures of human monocytes/macrophages infected with MBG 9 days p.i. (A) Overview of a segment of an infected monocyte/macrophage showing viral particles in multiple cytoplasmic vacuoles (arrowheads). Budding of viral particles was observed from the plasma membrane (B) (arrow) and membranes surrounding intracellular cytoplasmic vacuoles (C) (arrows). V, viral particle. Bars, 0.5 μm.

were found exclusively in the perinuclear region of the infected cells (Fig. 1). The first signs of cytopathogenic effects with cells rounded off were observed 5 to 6 days p.i. Examination of infected monocytes/macrophages by transmission electron microscopy revealed numerous viral particles in intracellular cytoplasmic vacuoles. The vacuoles differed greatly in number and size (Fig. 2A). Virus budding was observed at the plasma membrane (Fig. 2B) and at the membrane surrounding the vacuoles (Fig. 2C). The structural variability of intra- and extracellular viral particles was consistent with previous descriptions of MBG particles observed in human organs (35) as well as in infected Vero E6 cells and HUVECs (39). Both the number of infected cells and the cytopathogenic effect increased with time and resulted in cellular degeneration and disintegration in advanced stages (14 to 17 days p.i.). In contrast, mock-infected cultures remained viable and did not display any cell degeneration or disintegration at these time points (data not shown). Replication and release of viral particles were also analyzed by metabolic labeling and SDS-PAGE of MBG structural proteins, as well as reverse transcriptase PCR analysis of genomic RNA isolated from released viral particles. The infectivity of released MBG was determined by

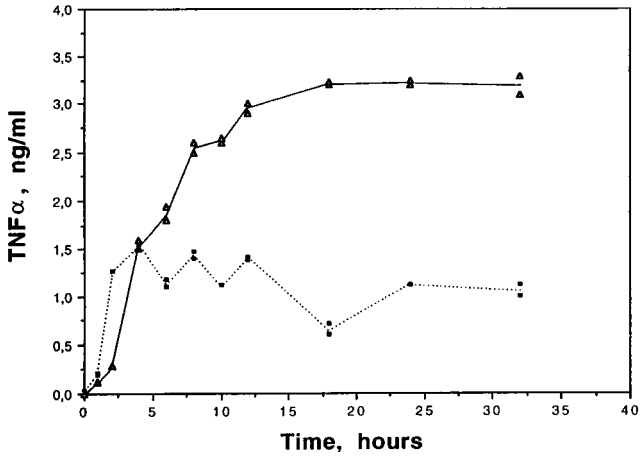

FIG. 3. Time course of TNF-α release. Results were obtained with supernatants of MBG-infected (▲) and mock-infected (■) cultured human monocytes/macrophages.

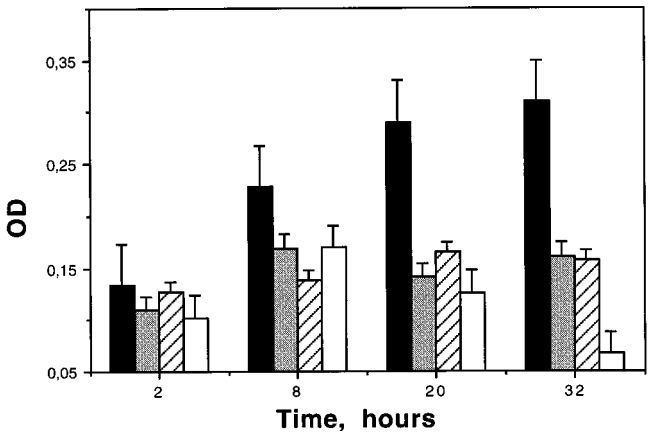

FIG. 4. Increased permeability of confluent cultures of HUVECs in response to culture supernatants of MBG and mock-infected human monocytes/macrophages. Solid bars, culture supernatants of MBG-infected monocytes/macrophages; shaded bars, culture supernatants of MBG-infected monocytes/macrophages after incubation with a neutralizing anti-TNF-α antibody (60 μg/ml); hatched bars, culture supernatants of mock-infected monocytes/macrophages; open bars, culture supernatants of mock-infected monocytes/macrophages after incubation with a neutralizing anti-TNF-α antibody. Note that the permeability-increasing effect of culture supernatants of MBG-infected monocytes/macrophages was abolished by addition of TNF-α-neutralizing monoclonal antibodies. OD, optical density at 470 nm.

a plaque assay in which virus was first detectable 24 to 32 h p.i. (data not shown).

Virus-induced activation of monocytes/macrophages. Activated monocytes/macrophages typically secrete various humoral factors, including cytokines, prostanoids, free oxygen radicals, etc. To examine whether monocytes/macrophages were activated by MBG infection, TNF-α was chosen as a prototype cytokine and important mediator of activated monocytes/macrophages. Tissue culture supernatants from MBG-infected and mock-infected cultured human monocytes/macrophages were collected at different time points p.i. (0 to 32 h) and assayed by ELISA for the presence of TNF-α. Twelve to 24 h p.i., supernatants of infected cultures reached peak values of biological, active TNF-α (~3 ng/ml) twofold higher than those observed in mock-infected cultures (Fig. 3).

Tissue culture supernatants of MBG-infected monocytes/macrophages increase paraendothelial permeability. To investigate the effect of tissue culture supernatants of MBG-infected monocytes/macrophages on endothelial permeability, we used an in vitro transwell filter assay in which the passage of HRP through an endothelial cell monolayer (grown on a filter) is assayed (22, 23). A significant increase in permeability was detected with culture supernatants of infected monocytes/macrophages collected 4 h p.i. A maximum of increase in permeability of approximately 120% was reached with culture supernatants obtained 20 and 32 h p.i. (Fig. 4). The time course of the potency of culture supernatants to increase endothelial permeability correlated with the increase of TNF-α release of infected monocytes/macrophages (Fig. 3). The permeability-increasing effect was abolished by the addition of a TNF-α-specific monoclonal antibody (Fig. 4). To exclude the possibility that the increased permeability was due to viral replication in endothelial cells and subsequent cytolysis, endothelial cell monolayers were infected with MBG (MOI of 1 to 10 PFU per cell) and assayed for HRP filtration under identical conditions. Within 48 h p.i., no significant increase in permeability could be detected (data not shown).

In order to confirm the causative role of TNF-α in mediating permeability, recombinant TNF-α was tested for its ability to increase endothelial permeability. Recombinant TNF-α at a concentration of 1 μg/ml caused a significant increase in endothelial permeability within 12 h (data not shown). This concentration of TNF-α is approximately 300 times higher than the concentrations measured in culture supernatants of MBG-infected monocytes/macrophages (~3 ng/ml). In the presence of 10 μM H_2O_2, however, a TNF-α concentration of 10 ng/ml, which is in the order of magnitude measured in culture supernatants of infected monocytes/macrophages, was sufficient to increase endothelial permeability (Fig. 5). H_2O_2 by itself, even at concentrations of 100 μM, did not increase endothelial permeability within 12 h.

Increased permeability is due to formation of interendothelial gaps. Confluent endothelial cell monolayers were exposed to culture supernatant of MBG-infected monocytes/macrophages. After 12 h of incubation, the cells were fixed and stained with an antibody directed to plakoglobin. Plakoglobin is a component of the adherens-type junctions in epithelial and endothelial tissues. Plakoglobin is restricted to a noninterrupted continuous band along interendothelial junctions in situ (13) and in endothelial cultures (Fig. 6A). Plakoglobin has been shown to disappear from the interendothelial junctions in response to the loss of intercellular adhesion (21). Treatment of confluent endothelial cell monolayers with culture supernatants of MBG-infected monocytes/macrophages resulted in a streaked and dotted staining pattern along interendothelial cell borders, demonstrating a redistribution of plakoglobin. This was most pronounced when culture supernatants collected 32 h p.i. were tested (Fig. 6C). The redistribution of plakoglobin indicative of interendothelial gap formation correlated with the increase in permeability. Similar results were obtained with recombinant TNF-α (10 ng/ml) in the presence of 10 μM H_2O_2 (Fig. 6D). Control monolayers treated with mock-infected supernatants (Fig. 6B), H_2O_2 (100 μM), or concentrations of TNF-α not capable of enhancing permeability in confluent cultures of endothelial cells, did not show a redistribution of plakoglobin, and thus gap formation was not induced.

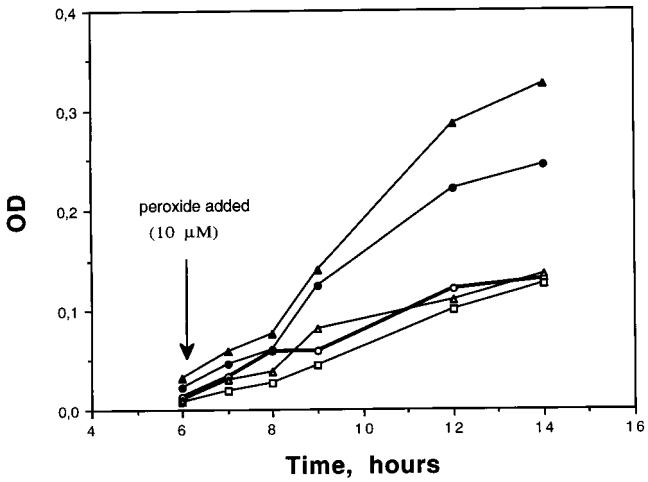

FIG. 5. Time course of the effect of recombinant TNF-α and H_2O_2 on endothelial permeability in confluent cultures of HUVECs. ○, no TNF-α and no H_2O_2; □, no TNF-α and 10 μM H_2O_2; △, 1 ng of TNF-α per ml and 10 μM H_2O_2; ●, 10 ng of TNF-α per ml and 10 μM H_2O_2; ▲, 100 ng of TNF-α per ml and 10 μM H_2O_2. OD, optical density at 470 nm.

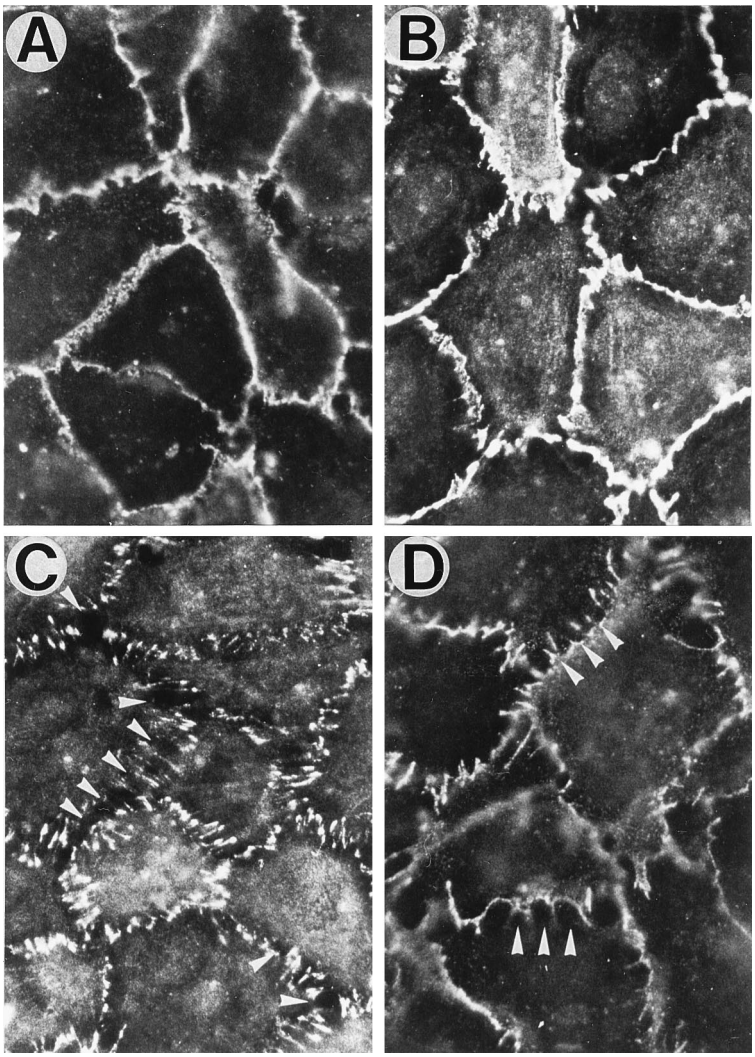

FIG. 6. Visualization of interendothelial gap formation in confluent cultures of HUVECs by immunofluorescence staining with plakoglobin antibodies. A continuous band of plakoglobin immunoreactivity is seen in untreated cultures (A) and in cultures treated for 12 h with supernatants of mock-infected monocytes/macrophages (B) (obtained 32 h p.i.). Gap formation and disturbances of the continuous staining pattern for plakoglobin (arrowheads) are seen in endothelial cell cultures treated for 12 h with supernatants of MBG-infected monocytes/macrophages (C) (obtained 32 h p.i.) and in endothelial cell cultures treated for 12 h with 10 ng of recombinant TNF-α per ml and 10 μM H_2O_2 (D).

DISCUSSION

The mechanisms underlying DIC, hemorrhage, and shock seen in severe or fatal courses of filovirus HF infection are poorly understood. In the present study, we examined in vitro the susceptibility of human monocytes/macrophages to infection with MBG, their response to infection, and the capability of infected monocytes/macrophages to enhance permeability in endothelial cell monolayers.

Human monocytes/macrophages are fully susceptible to infection and replication of MBG. This observation raises the possibility that infected monocytes/macrophages play an important role in generalization of the infection. Intracellular budding and accumulation of virus particles in vacuoles (Fig. 2) provide a mechanism by which virus hidden from the host's immune surveillance may spread. Virus particles could be passed to other cells of the body by exocytosis of the vacuoles or by release after cell lysis, as demonstrated in vitro. Our observations are in line with data obtained in vivo from filovirus-infected monkeys in which monocytes/macrophages are considered to be one of the primary target cells (14). A similar role of monocytes/macrophages was discussed earlier for human immunodeficiency virus (31) and Visna virus (32) infections. MBG infection of human monocytes/macrophages led to their activation, as demonstrated by the increased release of TNF-α, which is one of several cytokines typically secreted by activated monocytes/macrophages (Fig. 3). TNF-α has been previously shown to interact with specific endothelial cell receptors (29) and to trigger increased endothelial permeability (6, 18, 23), loss of anticoagulant activity (37, 38), and expression of adhesion molecules for leukocytes and platelets (4, 44). Furthermore, it has been demonstrated that TNF-α causes hemorrhage in several organs of the rat when infused at milligram quantities (42) and that TNF-α is involved in shock development in response to endotoxin (6). One of the most important events during shock development is the increase in paraendothelial permeability, in which TNF-α appears to play an important role as a mediator (17). In this study, we showed that filovirus-induced activation of monocytes/macrophages re-

sulted in the release of mediators into culture supernatants that cause an increase in paraendothelial permeability. The time course of TNF-α release measured in infected culture supernatants (Fig. 3) correlated with the ability of supernatants to increase endothelial permeability (Fig. 4). The release seemed to be triggered early in infection of monocytes/macrophages, long before cell damage and cytolysis occurred. This is supported by the time course of the permeability effect and the TNF-α release (Fig. 3 and 4).

Concentrations of TNF-α in serum in clinical cases of infection, including those with fulminant endotoxin and cardiac shock, were relatively low (~0.33 ng/ml) and never exceeded 4 ng/ml (18). Likewise, in our study, peak values of TNF-α measured in tissue culture supernatants of MBG-infected monocytes/macrophages reached ~3 ng/ml (Fig. 3). On the other hand, it was shown in vitro that endothelial permeability did not increase in response to TNF-α concentrations below 4 ng/ml (18). The data presented here support the notion that several secretory products (e.g., TNF-α, gamma interferon, macrophage-specific interleukins, proteases, and oxygen radicals) are released from virus-infected monocytes/macrophages and that the effect on permeability is cumulative. This assumption has been confirmed by data recently published by Ishii et al. (18) and obtained here showing that even at low concentrations (3 ng/ml), which had no direct permeability-increasing effect, TNF-α was capable of priming the effect in the presence of other factors or agents such as interleukin 2 and H_2O_2 (18) (Fig. 5). TNF-α, however, seems to play a critical role in mediating paraendothelial permeability, as demonstrated by the inhibitory effect of anti-TNF-α antibodies (Fig. 4).

The combination of viral replication in endothelial cells (14, 39) and virus-induced cytokine release from monocytes/macrophages may also promote a distinct proinflammatory endothelial phenotype that then triggers the coagulation cascade and, as a result, the development of DIC (3, 4, 37). This is of particular interest for filoviral HF, since the development of DIC is a complicating event in the final stages of the disease (28, 33). Hemorrhagic manifestations and generalized shock in prefinal stages of severe filoviral HF cases typically develop within 10 to 20 days p.i. (28, 33). The onset of these symptoms late in infection might be due to an uncontrolled virus-induced lysis of monocytes/macrophages resulting in a sudden massive release of secretory products that affect the integrity of the microvascular wall. This is supported by the observation that virus-induced macrophage lysis in vitro occurred within 14 to 17 days p.i.

Filovirus glycoproteins contain a region which shows significant homology to a domain in retroviral envelope proteins that has been suspected to be responsible for the immunosuppressive properties of these viruses (5, 36, 43, 45). In the retrovirus system, this domain seems to be involved in downregulation of certain immune functions of the cellular response. An immunosuppressive function of this domain, the infection of monocytes/macrophages in vivo (14) and in vitro (demonstrated here), and the virus-induced release of cytokines are in line with an immune response depression found in Ebola virus-infected monkeys.

Studies to understand the mechanisms leading to viral HF have so far not appropriately addressed the final steps that cause hemorrhage, shock, and sudden death. Data presented here provide the first experimental evidence for a virus-induced increase in endothelial permeability. They further suggest that the course of the disease will depend on the extent of macrophage and endothelial cell infection and on the effect on virus-induced release of humoral factors, such as cytokines. Recent studies of Argentine hemorrhagic fever, caused by Junin virus, a member of the family *Arenaviridae*, reported increased TNF-α values (up to 0.1 ng/ml) in acute-phase sera of patients, indicating virus-induced activation of leukocytes. In these cases, TNF-α levels correlated with the severity of the disease (16). In addition, the characteristic prominent pulmonary involvement in patients with hantavirus pulmonary syndrome, which is causally associated with Sin Nombre virus (of the family *Bunyaviridae*), is most likely caused by an increased vascular permeability. The increased permeability in this syndrome has been suggested to be caused by products released from leukocytes (e.g., monocytes/macrophages) and/or by a direct effect of virus infection on endothelial cells (7).

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