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Virology 321 (2004) 1–7
VIROLOGY
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Rapid Communication

Bovine aortic endothelial cells are susceptible to Hantaan virus infection

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Received 18 November 2003; returned to author for revision 19 January 2004; accepted 21 January 2004

Abstract

Hantavirus serotype Hantaan (HTN) is one of the causative agents of hemorrhagic fever with renal syndrome (HFRS, lethality up to 10%). The natural host of HTN is *Apodemus agrarius*. Recent studies have shown that domestic animals like cattle are sporadically seropositive for hantaviruses. In the present study, the susceptibility of bovine aortic endothelial cells (BAEC) expressing $\alpha_V\beta_3$-integrin to a HTN infection was investigated. Viral nucleocapsid protein and genomic RNA segments were detected in infected BAEC by indirect immunofluorescence assay, Western blot analysis, and reverse transcription-polymerase chain reaction (RT-PCR), respectively. The results of this study strongly support our previous observation on Puumala virus (PUU) that has been propagated efficiently in BAEC. These findings open a new window to contemplate the ecology of hantavirus infection and transmission route from animal to man.
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Keywords: Hemorrhagic fever with renal syndrome; *Bunyaviridae*; Hantavirus; Hantaan virus; Puumala virus; Bovine aortic endothelial cell

Introduction

Hantaviruses are enveloped, cytoplasmic RNA viruses with a single-stranded, negative-sensed RNA genome that consists of three segments [small (S), medium (M), and large (L)] that are coding for the nucleocapsid protein (N), two envelope glycoproteins (G1, G2), and the viral RNA-dependent RNA polymerase, respectively (Tidona and Darai, 2002; van Regenmortel et al., 2000). Hantavirus serotype Hantaan (HTN) was isolated in 1978 by Ho Wang Lee from lung tissue of the striped field mouse *Apodemus agrarius* (Lee et al., 1978) that is distributed in large parts of Asia and in the southeast of Europe. HTN is the prototype species of the genus *Hantavirus* of the family *Bunyaviridae* (Schmaljohn and Dalrymple, 1983) and is in addition to serotypes Seoul, Dobrava, and Puumala, the causative agent of hemorrhagic fever with renal syndrome (HFRS), a human disease characterized by severe renal failure, hemorrhages and shock (Lee et al., 1990). HFRS affects more than 200 000 people each year in Europe and Asia with a lethality up to 10% (Lee et al., 1990) as far as HTN is concerned. In contrast, the New World hantaviruses such as serotypes Sin Nombre, Black Creek Canal, Bayou, Laguna Negra, Rio Marmore, and Andes are causing the Hantavirus Pulmonary Syndrome (HPS) in humans characterized by a lethality of >50% (Schmaljohn and Hjelle, 1997). The main targets for hantaviral replication are diverse cell types of the endothelium (Kanerva et al., 1998) without causing an apparent cytopathic effect (Green, 1998). The susceptibility of human vascular endothelial cells to pathogenic and nonpathogenic hantaviruses had been reported by Yanagihara and Silverman in 1990. Human umbilical vein endothelial cells were infected with several strains of hantaviruses. Inclusion bodies and cytopathic effect were not observed. Hantavirus-specific intracytoplasmic perinuclear granular fluorescence was detected at 3 days postinfection. Ten days after initial infection, nearly 100% of cells contained viral antigen (Yanagihara and Silverman, 1990). These data led to the conclusion that endothelial cells may serve as target cells in hemorrhagic fever with renal syndrome. The major criterion of acute HFRS and HPS is altered vascular permeability of

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doi:10.1016/j.virol.2004.01.017

the infected endothelium of kidney or lung, respectively (Geimonen et al., 2002).

Each hantavirus serotype has its own specific rodent species as natural host and is distributed within the environment of its host spreading to humans via urine, feces, and saliva by inhalation or even by bites (Mills and Childs, 2001). Evidence of an infection with Sin Nombre virus (SNV) in domestic animals was reported by Malecki et al. (1998). Sera of 145 cats, 48 dogs, 120 horses, and 24 cattle were tested by Western blot analysis using SNV nucleocapsid antigen. This study revealed that 4 out of 145 (2.8%) cats and 4 out of 85 (3.5%) dogs had trace reactivity to full-length SNV-encoded nucleocapsid protein (Malecki et al., 1998). Furthermore, in the Czech Republic, Danes et al. (1992) found an antibody response against Puumala virus (PUU) in 3.5% of examined hares and in 14.1% of examined deer species. In addition, it was reported that 1.4% (two out of 145) cattle (*Bos taurus*) were found to possess antibodies against the serotypes Hantaan and Puumala (Danes et al., 1992). It is conceivable that hantaviruses are not only able to infect rodents and humans but also other animals especially domestic animals. This could represent an up to now underestimated route of transmission of this virus group to humans. The role, which domestic animals are playing in the ecology of hantaviruses, is still unknown.

Recently, we succeeded to propagate hantavirus serotype Puumala in bovine aortic endothelial cell (BAEC) cultures (Muranyi et al., 2004). This observation raised the question if the ability of Puumala virus to infect BAEC is a common feature of all hantaviruses or a specific property of this particular virus. In the present study, we demonstrate the susceptibility of BAEC to a hantavirus serotype Hantaan infection. The results of these studies are of particular interest because they confirm the universality of the finding that BAEC are susceptible to the infection of different hantavirus serotypes. Furthermore, they open a new window for analysis and understanding the underlying mechanisms influencing the ecology of hantaviruses.

Results and discussion

To investigate the susceptibility of BAEC to hantavirus serotype Hantaan, freshly grown monolayers of BAEC cultures were inoculated with Hantaan virus strain 76-118. The infected BAEC cultures were incubated for 2 h at 37 °C, washed three times with BME, refed with BME-FC-10, and incubated at 37 °C in a 5% CO_2 atmosphere. Two weeks later, the infected cell cultures were trypsinized and reseeded (1:3 dilution) in plastic culture flasks or plates. The further cell culture passages were carried out in 3-week intervals. The cell cultures were weekly refed with BME-FC-10. Daily microscopical observation of infected BAEC cultures revealed no evidence for a cytopathic effect (CPE) and showed no morphological alterations in comparison to mock-infected BAEC cultures.

Table 1
Detection of nucleocapsid protein of hantavirus serotype Hantaan strain 76-118 in comparison to serotype Puumala strain CG18-20 in infected and mock-infected BAEC and Vero E6 cultures at different cell culture passages by indirect immunofluorescence assay

	Cell culture	Virus	First passage (2 weeks p.i.)		Second passage (5 weeks p.i.)	Third passage (8 weeks p.i.)	Fourth passage (11 weeks p.i.)	Sixth passage (17 weeks p.i.)
			15 days p.i.	18 days p.i.				
A	BAEC	Mock	–	–	–	–	–	–
	BAEC	Hantaan 76-118	+/–	5%	25%	90%	100%	100%
	BAEC-Val-L 9.2	Mock	–	–	–	–	–	–
	BAEC-Val-L 9.2	Hantaan 76-118	–	+/–	10%	75%	90%	100%
	Cell culture	Virus	First passage (2 weeks p.i.)	Second passage (6 weeks p.i.)	Third passage (10 weeks p.i.)	Fourth passage (12 weeks p.i.)	Twelfth passage (6 months p.i.)	
B[a]	BAEC	Puumala CG18-20	–	–	20%	95%	100%	
	BAEC	Puumala Sotkamo	–	+/–	20%	90%	100%	
	BAEC-Val-L 9.2	Puumala Sotkamo	–	–	20%	100%	100%	
	Cell culture	Virus	First passage (2 weeks p.i.)		Second passage (4 weeks p.i.)	Third passage (6 weeks p.i.)	Fourth passage (8 weeks p.i.)	Sixth passage
			7 days p.i.	21 days p.i.				
C[b]	Vero E6	Mock	–	–	–	ND	ND	ND
	Vero E6	Hantaan 76-118	2%	5%	50%	>95%	ND	ND
	Vero E6	Puumala CG18-20	–	2%	20–30%	75%	>95%	ND

p.i. post infection (after initial infection).
ND not done.
−, no viral nucleocapsid protein detected.
+/−, unclear status of viral nucleocapsid protein presence.
%, percentage of cells in the culture that are positive for viral nucleocapsid protein.
[a] For comparison, a part of data obtained from our previous report on susceptibility of BAEC to Puumala virus (Muranyi et al., 2004) is given.
[b] For comparison, the results of an analogous experiment in which the Vero E6 cell cultures were used for propagation of Hantaan virus strain 76-118 and Puumala virus strain CG18-20 are shown.

The infected and mock-infected BAEC cultures were screened routinely for detection of Hantaan virus nucleocapsid protein by indirect immunofluorescence assay (IFA) to assess the extent of viral infection and replication. Three weeks after initial infection, Hantaan virus nucleocapsid antigen was detectable in 5–10% of infected BAEC increasing to 100% at the fourth cell culture passage and the results are summarized in Table 1. An example of IFA visualizing the typical perinuclear aggregation of nucleocapsid protein is shown in Fig. 1, Part A.

Confirmation of the aforementioned results was provided by repeating the infection experiment with the bovine aortic endothelial cell line BAEC-Val-L 9.2 showing no funda-

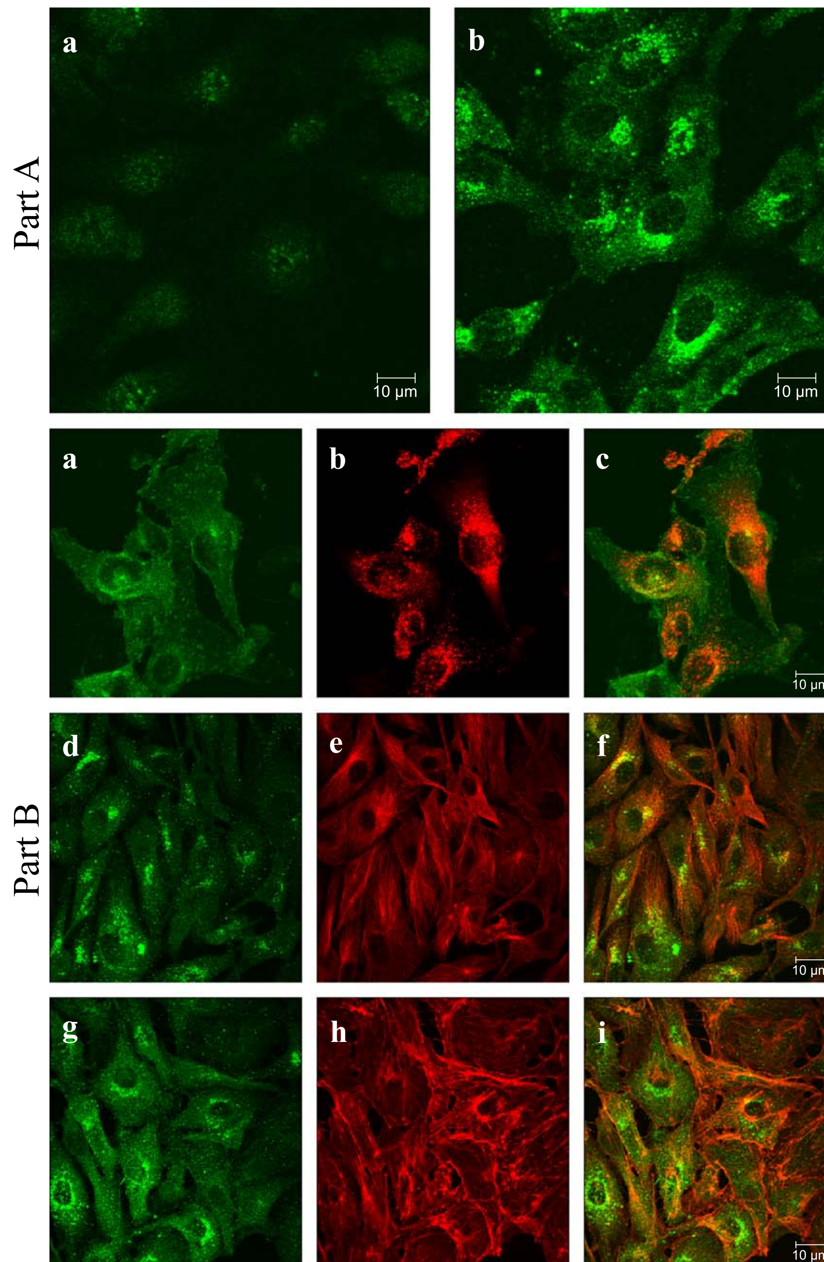

Fig. 1. (Part A) Indirect immunofluorescence assay (IFA) of hantavirus serotype Hantaan strain 76-118-infected bovine aortic endothelial cells (BAEC) for detecting viral nucleocapsid protein. A control IFA with a mock-infected BAEC culture is shown in panel a. Panel b displays the typical perinuclear aggregation of hantaviral nucleocapsid protein (green fluorescence) in about 90% of visualized cells of an infected BAEC culture at the third passage (10 weeks after initial infection). (Part B) Detection of Hantaan virus nucleocapsid protein, $\alpha_V\beta_3$-integrin, microtubules, and actin filaments of a BAEC culture productively infected with Hantaan virus at the first cell culture passage (3 weeks after initial infection). The Hantaan virus nucleocapsid protein was detected in typical perinuclear aggregation in the cells of the infected BAEC culture [panels b (red fluorescence), d, and g (green fluorescence)]. Cytoskeleton components like actin fibers (panel h) and microtubules (panel e) as well as $\alpha_V\beta_3$-integrin (panel a) of infected BAEC are detected by red and green fluorescences, respectively. Panels c, f, and i represent merged images of the viral nucleocapsid protein fluorescence and the $\alpha_V\beta_3$-integrin, microtubules, and actin fluorescences, respectively.

Fig. 2. Detection of Hantaan virus strain 76-118 and Puumala virus strain CG18-20 nucleocapsid protein in infected BAEC cultures separated by SDS-PAGE (12%), electroblotted, and developed by immunoblot analysis. The corresponding Coomassie blue staining is shown in panel A. The immunoblot was performed using rabbit antisera (diluted 1:1000) raised against recombinant nucleocapsid protein of Hantaan strain 76-118 (panel B) or against Puumala virus strain CG18-20 (panel C). The alkaline phosphatase-conjugated antibodies (anti-rabbit Ig-AP) were used in a dilution of 1:2000. Lanes: 1, molecular weight markers; 2, buffer only; 3, mock-infected BAEC; 4, buffer only; 5 and 6, persistently infected BAEC with Puumala virus (BAEC-PUU) at the seventh cell culture passage (lane 5) and eighth cell culture passage (lane 6); 7, buffer only; 8, infected BAEC with Hantaan virus at the fourth cell culture passage; 9, buffer only; 10, molecular weight markers. The arrows mark the positions of Hantaan virus (48 kDa) and Puumala virus (52 kDa) nucleocapsid proteins.

mental deviations in comparison to the application of the BAEC line (Table 1, Part A).

The efficiency of HTN infection on BAEC cultures was compared to the data of the previous study in which the Puumala virus was used to infect BAEC cultures (Muranyi et al., 2004; Table 1, Part B). The major difference between both hantavirus serotypes was found to be the incubation time of viruses before detection of specific hantavirus nucleocapsid protein in infected cells by IFA. Furthermore, it was found that the virus titer in cell-free supernatant of HTN propagated on BAEC is about one to two log units higher when compared to the titer of Puumala virus serotype under the same conditions. This is in agreement with the data known for efficiency of Hantaan and Puumala virus replication on Vero E6 cell cultures (Zöller et al., 1989). In addition, the efficiency of Vero E6 cell cultures as standard cell system for propagation of hantaviruses were compared to the data obtained using BAEC. As shown in Table 1, Part C, the efficiency of Vero E6 cell culture for propagation of hantaviruses is much higher in comparison to the BAEC cultures.

A more detailed characterization of Hantaan virus–host cell interactions was performed by IFA detection of viral nucleocapsid protein, microtubuli, actin filaments, and $\alpha_V\beta_3$-integrin distribution in infected BAEC cultures. An intact cytoskeleton structure is of indispensable importance for the function of BAEC and therefore it was examined whether the infection interferes with the integrity of the cytoskeleton. Typical perinuclear aggregation of Hantaan virus nucleocapsid protein in infected cells at the third cell culture passage is shown in Fig. 1, Part B, panels b–d, f, g, and i. Infected cells revealed no severe alteration of actin filaments (Fig. 1, Part B, panels h and i). Despite the highly productive infection, the microtubules showed no depolymerization (Fig. 1, Part B, panels e and f). The results provide no indication of a damage of the cytoskeleton structure in Hantaan-infected BAEC. It is known that the cellular entry of pathogenic hantaviruses is mediated by $\alpha_V\beta_3$-integrin (Gavrilovskaya et al., 1999), an abundant surface receptor on various endothelial cells and thrombocytes (Cheresh, 1987; Hynes, 1992), which is also detectable on the surface of BAEC as shown in Fig. 1, Part B, panels a and c. In addition to IFA, the expression of the Hantaan virus nucleocapsid protein in infected BAEC cultures at the fourth passage was determined by Western blot analysis (Fig. 2, panels B and C, lane 8). The one-way cross-reactivity of HTN and Puumala virus is illustrated in Fig. 2, panel C, lane 8, in which the antiserum against Puumala virus was able to react weakly with the nucleocapsid protein of Hantaan virus. In contrast, the antiserum against HTN was unable to detect nucleocapsid protein of persistently infected BAEC-PUU as shown in Fig. 2, panel B, lanes 5 and 6) (Zöller et al., 1989).

To detect the presence of Hantaan virus RNA genome segments in infected BAEC cultures, RT-PCR analysis was carried out and the results are shown in Fig. 3. Specific RT-PCR products of 878, 590, and 519 bp for the viral RNA segments S, M, and L, respectively, were amplified from RNA that was isolated from cell-free supernatant of Hantaan virus-infected BAEC cultures at the fourth cell passage after

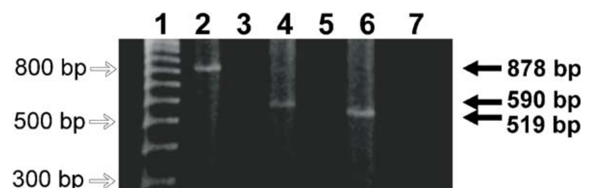

Fig. 3. Polyacrylamide gel electrophoresis showing the results of the RT-PCR experiments performed to determine the presence of Hantaan virus strain 76-118 S segment (lanes 2 and 3), M segment (lanes 4 and 5), and L segment (lanes 6 and 7) in cell-free supernatant of infected BAEC at the fourth cell culture passage. Lane 1 represents 100-bp DNA molecular marker. The results of RT-PCR using RNA extracted from cell-free supernatant of infected BAEC are shown in lanes 2, 4, and 6 for S, M, and L viral RNA segments of Hantaan virus strain 76-118, respectively. The results of the corresponding control experiments (PCR without reverse transcription step to exclude DNA contamination) are given in lanes 3, 5, and 7 for S, M, and L viral RNA segments of Hantaan virus strain 76-118, respectively. The bold arrows represent the sizes of the amplified RT-PCR products in base pairs.

initial infection. An eventual DNA contamination in the RNA preparations was excluded by control PCR reactions without the reverse transcription step.

To prove whether the HTN-infected BAEC cultures release infectious virus into the culture medium, the cell-free supernatant of HTN-infected BAEC cultures was examined. Monolayers of BAEC cultures grown in six-well plate (35 mm in diameter/well) were infected by addition of 1 ml cell-free supernatant per well obtained from HTN-infected BAEC cultures at the fourth cell passage after initial virus infection in which nearly 100% of cells were infected as tested by indirect IFA. The infected cells were incubated at 37 °C for 2 h, washed three times with BME, refed with BME-FC-10, and incubated at 37 °C in a 5% CO_2 atmosphere. The subsequent analysis of the infected cells by IFA and immunoblotting revealed that the cell-free supernatant of HTN-infected BAEC cultures was able to infect freshly grown BAEC. An example of the results obtained by immunoblot analysis is shown in Fig. 4. The results of these studies indicate that infected cells shed infectious virus particles into the cell culture medium and the state of HTN infection on BAEC is a productive infection. This is in agreement with the data obtained by infection of BAEC using Puumala virus (Muranyi et al., 2004).

Rodents seem to represent the major natural reservoir of hantaviruses and are regarded as the only infection source for humans (Meyer and Schmaljohn, 2000). However, the existence of hantavirus serotypes causing human diseases with a very high mortality makes it necessary to examine hantavirus ecology in more detail. The present and recent studies (Muranyi et al., 2004) showed that endothelial cells of bovine origin are susceptible to productive hantavirus infection raising the possibility that the host range of hantaviruses comprise more than only humans and rodent species. Domestic animals and rodents frequently live jointly in a similar habitat and transmission of hantaviruses from rodents to domestic animals seems to be conceivable representing an until now unexpected new transmission route to animals and possibly to man. The identification of seroprevalences for different hantavirus serotypes in cattle (Danes et al., 1992) and the detection of susceptibility of BAEC to HTN and Puumala virus infection (Muranyi et al., 2004) are new aspects in the ecology of these important human pathogens. One can assume that the ecology of hantaviruses in domestic animals is different from that known for rodents. Advanced research on this subject should focus attention on in vivo infection of domestic animals. Such studies should include screening of domestic animals especially in regions that are known for endemic hantavirus occurrence to assess the infection risk for humans that emanates from species that are until now not suspected to be a reservoir for hantaviruses.

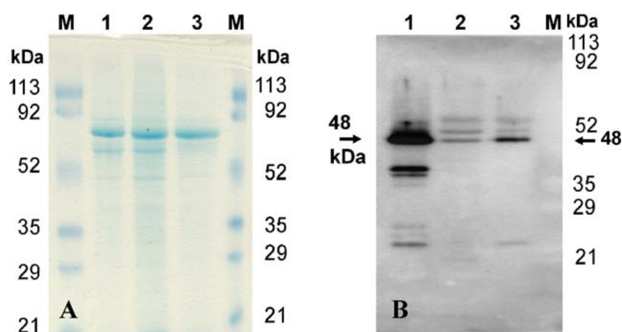

Fig. 4. Detection of Hantaan virus strain 76-118 nucleocapsid protein in infected BAEC cultures by Lumi-Light^{PLUS} Western blotting. The cellular proteins were separated by SDS-PAGE (12%), electroblotted, and developed by Kodak X-OMAT 1000 Processor, 2177 and Kodak X-OMAT, XAR-5. The corresponding Coomassie blue staining is shown in panel A. The immunoblot was performed using rabbit antisera (diluted 1:1000) raised against recombinant nucleocapsid protein of Hantaan strain 76-118 (panel B). The alkaline phosphatase-conjugated antibodies (anti-rabbit Ig-AP) were used in a dilution of 1:2000. Lanes: 1, HTN-infected BAEC at the fourth cell culture passage after initial infection; 2, the BAEC culture 3 weeks after infection with cell-free supernatant of the HTN-infected BAEC culture, and 3, the BAEC culture at the first cell culture passage after infection with cell-free supernatant of the HTN-infected BAEC culture. Molecular weight markers are given in lane M. The arrows mark the positions of Hantaan virus nucleocapsid proteins (48 kDa).

Materials and methods

Cell and virus

Bovine aortic endothelial cell cultures BAEC, BAEC-Val-L 9.2 (Nawroth et al., 1985; Schwartz, 1978), and persistently infected BAEC with Puumala virus (BAEC-PUU, Muranyi et al., 2004) were grown and propagated with Basal Medium Eagle (BME) and refed with BME supplemented with 10% fetal calf serum, 100 IE ml^{-1} penicillin G, 100 IE ml^{-1} streptomycin (BME-FC-10), and incubated at 37 °C in a 5% CO_2 atmosphere. Hantavirus serotype Hantaan strain 76-118 used in the study was propagated on Vero E6 cell cultures (ATCC, CCL-81) as described elsewhere (Welzel et al., 1998). The experimental infection with Hantaan virus was carried out in facilities of L3 laboratories at the University of Heidelberg.

Sera and antibodies

Antisera against recombinant nucleocapsid protein of hantavirus serotype Hantaan strain 76-118 and Puumala virus strain CG18-20 were induced in New Zealand white rabbits as described elsewhere (Muranyi et al., 2004). FITC-conjugated or Texas red-X-conjugated anti-rabbit IgG F(ab′)2 fragment immunoglobulin (Dianova, Hamburg, Germany) was used as secondary antibodies. For detection of $\alpha_V\beta_3$-integrin, a mouse monoclonal antibody was used and visualized by FITC-conjugated anti-mouse antibody. Cytoskeleton components were detected with a monoclonal antibody Mab DM 1A (anti-α-tubulin, Sigma) and phalloi-

din-FITC (anti-actin, Sigma) as described previously (Muranyi et al., 2004).

Indirect immunofluorescence assay and confocal microscopy

IFA was performed as described previously (Muranyi et al., 2004). BAEC cultures infected with Hantaan virus were fixed either with 3% paraformaldehyde or with methanol/acetone. Rabbit antisera raised against the nucleocapsid protein of HTN were used in a dilution of 1:1000. The mounted cells were analyzed with a laser scanning confocal microscope (Leica, Heerbrugg, Switzerland; microscope Leitz DM IRB; scanner Leica TCS NT).

Virus titer measurement

Due to the fact that the determination of virus titer of hantaviruses by plaque assay is not routinely practicable and reproducible, the infectivity of cell-free supernatant of infected cultures was determined by indirect immunofluorescence assay (IFA). Monolayers of Vero E6 cells freshly grown on coverslips (10 mm in diameter) in 24-well plates were infected with 100 μl of 10-fold dilution of individual virus probes (10^{-1} to 10^{-7}) and incubated for 2 h at 37 °C for virus adsorption. The cell cultures were washed two times with phosphate-buffered saline (PBS, pH 7.2) and supplemented with 2 ml BME-FC-10 and incubated at 37 °C. After 4, 6, and 8 days postinfection, the cells were washed three times with cold PBS and fixed in acetone/methanol (1:1) for 5 min at −20 °C. The fixed cells were preincubated in PBS containing 2.5% bovine serum albumin (BSA) for blocking nonspecific binding. The detection of HTN specific nucleocapsid protein was carried out using antibody dilution of 1:500 of rabbit antisera against recombinant nucleocapsid protein of hantavirus serotype Hantaan strain 76-118 and FITC-conjugated or Texas red-X-conjugated anti-rabbit IgG F(ab′)2 fragment immunoglobulin (Dianova) was used as secondary antibodies. The IFA was performed essentially as described earlier (Muranyi et al., 2004). The virus titer was estimated by measuring the value of nucleocapsid protein detected in each dilution used.

Immunoblot analysis

Confluent monolayers of cells were harvested by scraping the cells from the culture well, petri dishes, and flasks after being washed three times with PBS (pH 7.2). The final cell pellet was resuspended in distilled water. The electroblot onto nitrocellulose and subsequent staining with alkaline phosphatase-conjugated antibodies (anti rabbit Ig-AP, Boehringer Mannheim, Germany) was carried out as described previously (Muranyi et al., 2004).

The Western blot analysis for detection of the viral nucleocapsid protein was performed using the Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit) (Roche Diagnostics, Mannheim, Germany) as described previously (Muranyi et al., 2004). The goat anti-rabbit IgG (H + L) was used as second antibody at the dilution of 1:2500 (Dianova). The staining of the membrane was carried out using ECL^{TM} Western Blotting detection Reagents kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The exposition was performed by Kodak X-OMAT 1000 Processor, 2177 and Kodak X-OMAT, XAR-5.

RNA extraction

Hantavirus serotype Hantaan strain 76-118 was propagated on BAEC cultures and the viral RNA in the cell-free medium was isolated using the Qiagen viral RNA extraction kit as described elsewhere (Muranyi et al., 2004).

Reverse transcription-polymerase chain reaction

To determine the presence of specific Hantaan virus genome segments, oligonucleotide primers were constructed that encase specific regions of the cDNA sequences of the viral L [HAN-L1F: 5′-CAGTAGATGATGGGACTGACTGG-3′ nt 3363–3385); HAN-L1R: 5′-CAATCCCAGCTGTTGCTAACTCC-3′ (3881–3859)], M [HAN-M1F: 5′-AATGGGTTCAATGGTTTGTG-3′ (nt 1687–1706); HAN-M1R: 5′-CAGTGAAATGCAGTTTTAAG-3′ (nt 2275–2256)], and S RNA segment [HAN-S1F: 5′-GGCCAGACAGCAGATTGG-3′ (nt 376–393); HAN-S1R: 5′-AGCTCAGGATCCATGTCATC-3′ (nt 1253–1234)]. HAN-L1F, -M1F, and -S1F were used in the reverse transcription step. Reverse transcription-polymerase chain reaction (RT-PCR) experiments were performed and analyzed as described previously (Muranyi et al., 2004). The reverse transcription step was preceded by heating of RNA and oligonucleotide primers at 60 °C for 10 min. The PCR cycling conditions included an initial heating step of 95 °C for 10 min without Taq polymerase and a 35-cycle run under conditions of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min per cycle. RT-PCR products were purified by phenol/chloroform/isoamylalcohol extraction and sequenced using the DyeDeoxy Terminator *Taq* cycle sequencing technique (Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Weiterstadt, Germany) and a 373A "Extended" DNA sequencer (Applied Biosystems, Weiterstadt, Germany) as described previously (Muranyi et al., 2004; Tidona and Darai, 1997).

Acknowledgments

This study is dedicated to Professor Konrad Andrassy in occasion of his 65th birthday. The authors are grateful to Professor Peter Nawroth and Dr. Angelika Bierhaus for kindly providing the bovine aortic endothelial cells. The authors thank Dr. T. Chavakis for kindly providing the

mouse monoclonal antibody and FITC-conjugated anti-mouse antibody for integrin detection.

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