Arch Virol (2004) 149: 1279–1292
DOI 10.1007/s00705-004-0306-y

Archives of Virology
Printed in Austria

Modification of endothelial cell functions by hantaan virus infection: prolonged hyper-permeability induced by TNF-alpha of hantaan virus-infected endothelial cell monolayers

M. Niikura[1], **A. Maeda**[1], **T. Ikegami**[1,2], **M. Saijo**[1], **I. Kurane**[1], and **S. Morikawa**[1]

[1]Department of Virology 1, National Institute of Infectious Diseases, Musashimurayama, Japan
[2]Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Received November 26, 2003; accepted January 28, 2004
Published online March 25, 2004

Summary. Serious vascular leakage is central to the pathogenesis of hantavirus infections. However, there is no evidence suggesting the hantavirus infection of endothelial cells directly causes obvious cell damage or morphological alteration either *in vivo* or *in vitro*. In this study, we examined whether Hantaan virus (HTNV) infection modifies the barrier function of endothelial cell monolayers upon the exposure to pro-inflammatory cytokines. Low levels (1 ng/ml) of tumor necrosis factor-alpha initially increased the permeability in both HTNV-infected and uninfected monolayers similarly. Thereafter, however, these monolayers showed significant difference. The HTNV-infected monolayers remained irreversibly hyper-permeable during the experimental period up to 4 days, while the uninfected monolayers completely recovered the barrier function. The prolonged hyper-permeability of HTNV-infected monolayers was not associated with cell death or gap formation in the monolayers, and was independent from their nitric oxide or prostaglandin production. These results are the first evidence that hantavirus infection modifies barrier function of endothelial cell monolayers and suggest that HTNV-infection of endothelial cells may contribute to the increased vascular leakage through the prolonged response to cytokines.

Introduction

Hantaviruses belong to the family *Bunyaviridae* and are transmitted to humans from various species of rodents, which are the natural reservoirs. Hantaviruses

consist of numerous strains and each strain is maintained in different rodent species in a natural environment [31]. It is believed that the geographical isolation of these host species contributes to the regional variation in the virulence of hantaviruses [38]. The severest forms of the hantavirus infections are typified by hemorrhagic fever with renal syndrome (HFRS) mostly caused by isolates of *Hantaan virus* (HTNV) in eastern Asia and hantavirus pulmonary syndrome (HPS) caused by isolates of *Sin Nombre virus* (SNV), Andes virus and related viruses in Americas [6, 37, 38]. The major target organs appear to be different between these two forms of hantavirus infections: kidneys in HFRS and lungs and hearts in HPS. However, same organs are often affected in both diseases and it is considered that one of the fundamental pathological manifestations is vascular leakage [6, 7, 21, 37, 38].

Both HTNV and SNV infect endothelial and monocytic cells *in vitro* and *in vivo*. Neither hantavirus infections of cultured cells causes obvious cytopathic effects, contrary to some other hemorrhagic fever viruses like filoviruses [17, 36, 39, 43, 45]. In filovirus infection, for example, the disruption of endothelial cells *in vivo* directly relates to the massive vascular leakage [11]. An *in vitro* study showed that HTNV infection induced apoptotic endothelial cell death [16]. Nevertheless, histopathological examinations of either hantavirus-infected patients did not show major endothelial cell death [24, 35, 45]. Further, it was reported that SNV infection of endothelial cell monolayers did not alter the permeability or tight junction structures *in vitro* [17, 42]. It is, therefore, possible that both hantavirus infections affect the function of endothelial cells in a limited fashion.

HTNV-infected patients develop cellular and humoral immune responses before or very soon after the onset of clinical symptoms, leading to a hypothesis that the symptoms are partly due to immunopathogenesis [7]. Animal experiments with HTNV supported this hypothesis [18, 30, 34, 44]. Besides the virus-specific immunity, elevated levels of tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) in HFRS patients' sera were reported [22]. In an *in vitro* study, the infection of immature dendritic cells with HTNV weakly up-regulated TNF-alpha production [39]. Similarly, the infection of human alveolar macrophages with SNV *in vitro* resulted in a weak induction of TNF-alpha at around 1 ng/ml in the culture supernatant [17]. Endothelial cells carry two known TNF-alpha receptors, TNF-R75 and TNF-R55 [25, 27]. The effects of TNF-alpha on endothelial cells through these TNF-alpha receptors have been widely investigated. TNF-alpha increases vascular permeability [2, 5, 14, 33, 40]. It induces the release of cytokines and chemokines from endothelial cells [27]. It also up-regulates production of inducible enzymes such as cyclooxygenases-2 (Cox-2) and nitric oxide synthases (NOS) [4, 12, 28]. However, the relevance of these specific and non-specific immune responses elicited by hantavirus infections to their pathogenesis is not yet well understood.

Since neither the increase in permeability of endothelial cell monolayers [42] nor TNF-alpha induction in monocytic cells [17, 39] upon the hantavirus infections *in vitro* alone was accountable levels for the clinical manifestations seen in HFRS, we hypothesized that the low level of pro-inflammatory cytokines produced by HTNV-infected monocytic cells might differently affect HTNV-

infected and uninfected endothelial cells, due to possible functional alterations of the HTNV-infected endothelial cells. In order to test this hypothesis, we compared the responses of HTNV-infected and uninfected human umbilical vein endothelial cells (HUVEC) to low concentrations of cytokines.

Materials and methods

Cells and viruses

Pooled primary HUVEC was purchased from Clonetics (San Diego, CA). HUVECs were expanded and maintained in EGM-2 medium supplemented with growth factors and 2% bovine fetal serum (Bullet kit, Clonetics) as recommended by the supplier. The cells were cultured at 37 °C with 5% CO_2 throughout the experiments. HTNV strain 76–118 was provided by Dr. J. Arikawa (Hokkaido University, Japan) and propagated in Vero E6 cells.

Cytokines, antibodies and specific inhibitors

Recombinant human TNF-alpha was purchased from Lifetech (Rockville, MD). The specific activity was $>2 \times 10^7$ units/mg. Recombinant human IL-6 was purchased from Genzyme (Cambridge, MA). Neutralizing polyclonal antibodies specific to TNF-alpha and IL-6, respectively, were purchased from R&D systems (Minneapolis, MN). Neutralization of TNF-alpha prior to stimulation was achieved by mixing 100 ng/ml TNF-alpha and 50 μg/ml antibody and incubation on ice for 1.5 hr. Polyclonal rabbit antiserum to *Seoul virus* (a hantavirus species) strain SR-11 was prepared in our laboratory by inoculation of the live virus to a rabbit [41]. This antiserum was cross-reactive with HTNV. Monoclonal antibody specific to plakoglobin was purchased from Zymed (South San Francisco, CA). Specific inhibitors for NOS and Cox, aminoguanidine and L-NMA, and indomethacine, respectively, were purchased from Sigma (St. Lois, MO). They were used at the concentrations reported effective, respectively [8, 15, 28].

Preparation of HTNV-infected HUVEC

HUVECs were infected with HTNV at the passage level one and further passed twice. At the passage level of three in total, the HTNV-infected cells were frozen as aliquots in liquid nitrogen. Uninfected cells were similarly passed and frozen at the same passage level. The frozen cells were thawed and cultured in tissue culture flasks. These cells were trypsinized, counted and subjected to the experiments. The HTNV-infected and uninfected HUVECs were compared at the same passage levels between four and six. The percentage of HTNV-infected cells at the passage level four was greater than 50%, when determined by an immunofluorescent assay using the antiserum to Seoul virus. The absence of mycoplasma in both HTNV-infected and uninfected HUVECs was confirmed by a PCR-based kit (Mycoplasma Plus PCR Primer Set, Stratagene, La Jolla, CA).

Transmonolayer electrical resistance (TER) of HUVEC monolayers

TER of the HUVEC monolayers was examined by Endome chamber (World Precisions, Saratoga, FL). HUVECs were plated at a density of 1×10^5 cells per well in Transwell-col (0.33 cm^2 growth area, Coaster, Cambridge, MA) with 0.6 and 0.1 ml of the culture medium in the lower and upper chambers, respectively. The growth area was pre-coated with fibronectin according to the method by Bonner and O'Sullivan [3]. On the next day, the culture medium was carefully removed and the cells were re-fed with the same volumes of medium. Two or three days later, designated as time 0, the TER was measured in Endome chamber filled with

0.6 ml of the medium. All the reagents added to the wells were prepared in the medium as 100× concentrations to the final concentrations, and 1 μl was added to 0.1 ml of the medium present in the upper chambers. All the experiments were performed in triplicate and the results were expressed as averages and standard deviations (SD). The measured resistance was converted to net resistance per cm^2 according to the formula:

"Net resistance per cm^2"
= "[Measured resistance (ohm)-21(ohm, resistance without cell monolayer)]/3"

Permeability assay

Permeability of the HUVEC monolayers was examined as described previously [3]. Briefly, 1 g of bovine serum albumin (BSA, Sigma) was stained with 45 mg of trypan blue (Sigma) by mixing in 5 ml of the medium at room temperature (RT) for 2 hr. The binding of trypan blue to BSA was confirmed by precipitating the stained BSA by trichroloacetic acid. After centrifugation, the remaining free trypan blue in the supernatant was less than 0.07% in comparison with the pre-precipitation solution, when determined by the absorbance at 550 nm. The stained BSA was filter sterilized. To measure the leakage through cell monolayers, 10 μl of the stained BSA was added to the upper chamber of each Transwell containing HUVEC monolayer. After 4 hr of incubation, 100 μl of the medium in lower chambers was removed and the absorbance was measured at 550 nm.

Cell viability assay

Cell viability was examined by Cell Counting Kit-8 (CCK-8), which utilizes water-soluble tetrazolium salt (Dojin Chemical, Tokyo, Japan). The HTNV-infected and uninfected HUVECs were plated in 96-well tissue culture plates pre-coated with fibronectin, at a density of 1×10^5 cells per well in 200 μl of the medium. On the next day, the medium was completely replaced and cells were cultured for additional 2 days. Then TNF-alpha was added to the medium at a final concentration of 1 ng/ml. After 72 hr culture, 10 μl of the tetrazolium salt solution supplied in CCK-8 was added to each well and incubated for 1.5 hr. The culture supernatant (100 μl) was transferred to another 96-well plate and the absorbance was measured at 450 nm by a microplate reader with the reference wavelength at 600 nm.

Immunohistochemistry

The HTNV-infected and uninfected HUVECs were cultured on cover slips pre-coated with fibronectin in 48 well tissue culture plates at a density of 3×10^5 cells per well in 400 μl medium. Prior or after the TNF-alpha treatment, the cells were fixed in 4% formaldehyde at RT. The cells were permealized by 2% NP-40 and stained by anti-plakoglobin mouse monoclonal and anti-Seoul virus rabbit polyclonal antibodies. The reacted antibodies were detected by either FITC-labeled anti-mouse IgG (Zymed) or Rhodamine-labeled anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA), respectively.

Cytokine assays

IL-6, TNF-alpha and IL-1beta were quantitated by Quantikine ELISA kits (R&D Systems). The HTNV-infected and uninfected HUVECs (2.5×10^5 cells) were plated in 1 ml medium in 24 well tissue culture plates pre-coated with fibronectin. The medium was replaced on the

next day and cultured for additional 2 days. Before the addition of 1 ng/ml of TNF-alpha, the first sample was collected (time 0). After the indicated hours of culture, the supernatant (100 μl) was recovered from each well. The supernatants were kept at −80 °C until use.

Statistical analyses

Student's T-tests were performed using Microsoft Excel software.

Results

Effect of TNF-alpha on HTNV-infected endothelial cell monolayers

The transmonolayer electrical resistance (TER) of HUVEC monolayers was not changed by HTNV infection alone (Fig. 1A, time 0). The TER of HTNV-infected and uninfected monolayers was between 10 and 20 ohm/cm^2, which was comparable to the previous reports [2]. TNF-alpha significantly decreased the TER of HTNV-infected and uninfected monolayers at 1 ng/ml (Fig. 1A) and 10 ng/ml, but not at 0.1 ng/ml (data not sown). The HTNV-infected and uninfected monolayers showed similar levels of TER decrease as early as 4 hr after the addition of 1 ng/ml of TNF-alpha (Fig. 1A). The duration of the effect was, however, significantly different between the HTNV-infected and uninfected monolayers. In the uninfected monolayers, the decreased TER started to recover after 24 hr

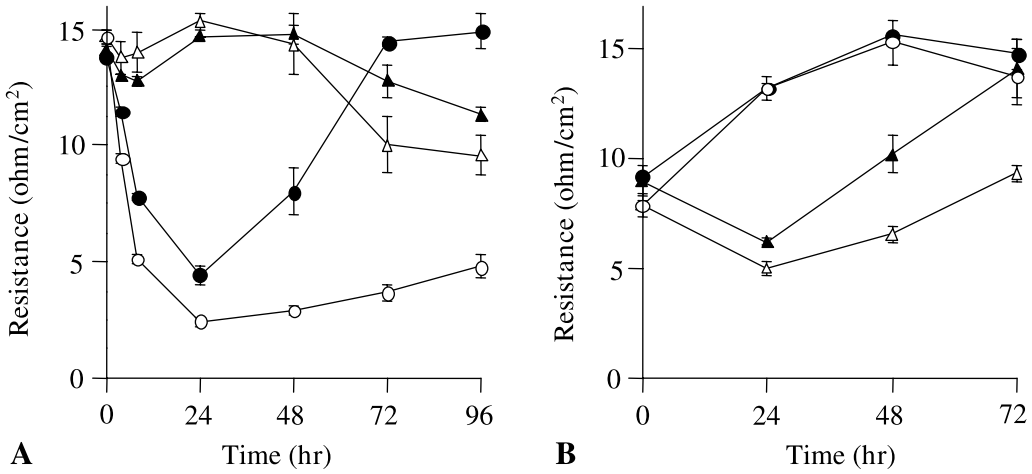

Fig. 1. Effect of TNF-alpha at 1 ng/ml on transmonolayer electrical resistance (TER) of HTNV-infected and uninfected HUVEC. **A**. HTNV-infected (○) and uninfected (●) monolayers were stimulated by TNF-alpha at time 0 and the TER was measured at 4, 8, 24, 48, 72 and 96 hr after stimulation. Unstimulated controls of HTNV-infected (△) or uninfected (▲) HUVEC monolayers were included. **B**. Contribution of TNF-alpha to the decreased TER after TNF-alpha stimulation. TNF-alpha was mixed with neutralizing antibody specific to TNF-alpha. After 1.5 hr incubation on ice, the neutralized (○, infected; ●, uninfected) or mock-neutralized (△, infected; ▲, uninfected) TNF-alpha was added to the HUVEC cultures. The TER was measured at 4, 8, 24, 48 and 72 hr after stimulation. Each point represents the mean value of triplicate. Bars indicate SD

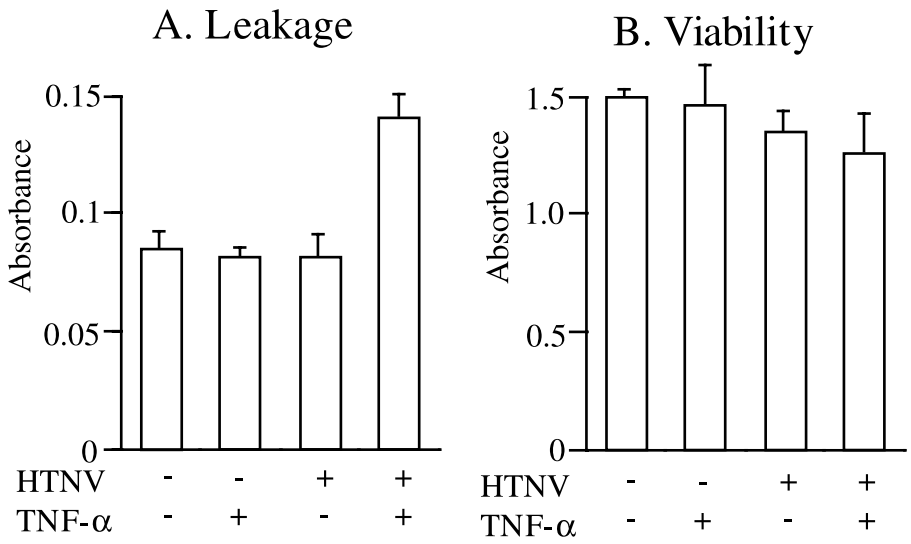

Fig. 2. A. Compatibility of decreased TER with increased permeability. Influx of stained BSA through the HTNV-infected (HTNV+) or uninfected (HTNV−) HUVEC monolayers was quantitated by the absorbance at 48 hr after TNF-alpha stimulation. The TER (average ± SD) at this time point for uninfected unstimulated, uninfected stimulated, infected unstimulated and infected stimulated monolayers were 13.8 ± 0.51, 20.3 ± 0.88, 15.8 ± 0.51 and 8.3 ± 0.33, respectively. **B**. Increased permeability was not due to cell death. Viabilities of HUVEC were examined at 72 hr after TNF-alpha stimulation (1 ng/ml) by the tetrazolim salt color development and shown as the absorbance of each supernatant. Each value represents the mean value of triplicate. Bars indicate SD

and reached the control level within 72 hr. Contrary, the decreased TER did not recover to the untreated level in the HTNV-infected monolayers even at 96 hr. The TER difference between uninfected and HTNV-infected monolayers at 72 hr after stimulation was statistically significant ($p < 0.01$). This decrease of TER was truly induced by TNF-alpha, since the anti-TNF-alpha neutralizing antibody completely abolished the decrease (Fig. 1B). The anti-TNF-alpha antibody itself showed no effect on the TER (data not shown).

To examine if the decreased TER actually reflected the increase in the permeability of monolayers, passive diffusion of BSA through the monolayers was examined (Fig. 2A). At 48 hr, when the HTNV-infected and uninfected monolayers showed significantly different TER ($p < 0.01$), the influx of stained BSA through the infected monolayers was significantly increased ($p < 0.05$) compared to the uninfected ones, indicating that the TER truly reflected the permeability. Further, the increase in permeability was not due to major cell death, since cell viability of these monolayers was not significantly different ($p > 0.05$) between the HTNV-infected and uninfected ones at 72 hr (Fig. 2B).

At any time point of TNF-alpha treatment, the peripheral localization of plakoglobin, which is a sensitive indicator for the matured endothelial cell-to-cell junctions [20], was not disturbed, suggesting that the junction structure was not destroyed by HTNV infection or this TNF-alpha treatment (Fig. 3). These results

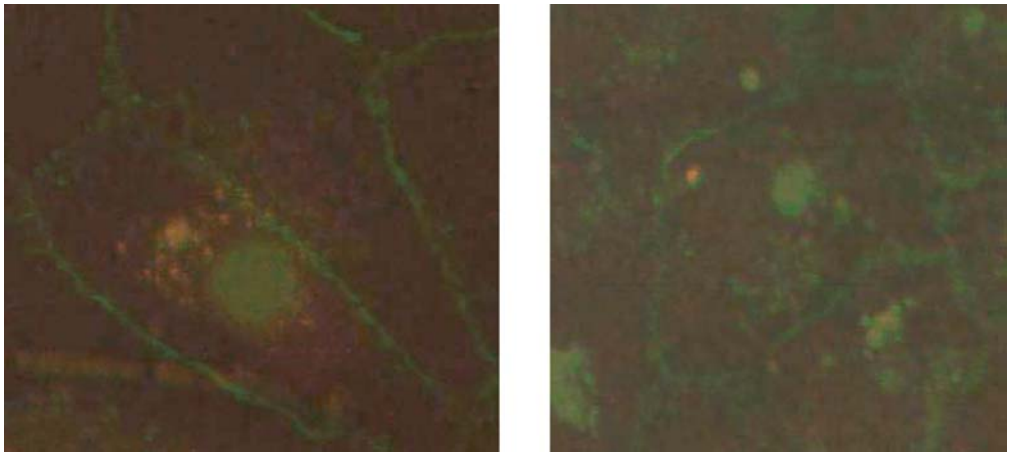

Fig. 3. Maintenance of cell-to-cell junction structures after TNF-alpha stimulation. HUVEC monolayers were stained by anti-plakoglobin and anti-Seoul virus (cross-reactive with HTNV) antibodies then visualized with FITC (green)- or Rhodamine (red)-labeled secondary antibodies, respectively. **A** HTNV-infected HUVEC at 72 hr after TNF-alpha stimulation. **B** Uninfected non-stimulated control HUVEC at 72 hr

indicate that the HTNV-infected and uninfected HUVEC monolayers show similar levels of initial hyper-permeability without major cell death upon the exposure to TNF-alpha, but the increased permeability remains for a prolonged period in the HTNV-infected monolayers.

Effect of antagonists to NOS and Cox on TNF-alpha-induced hyper permeability

It is known that TNF-alpha induces both nitric oxide (NO) and prostaglandin (PG) through the induction of NOS and Cox-2, respectively. We examined whether NO and PG were involved in the different time courses of the response between HTNV-infected and uninfected monolayers. Aminoguanidine and L-NMA are specific inhibitors for inducible NOS (iNOS) and endothelial NOS (eNOS), respectively. As shown in Fig. 4A and B, neither aminoguanidine (100 μM) nor L-NMA (1 mM) altered the responses of HTNV-infected and uninfected monolayers to 1 ng/ml of TNF-alpha. When 30 μM of indomethacine, a Cox inhibitor was added, TNF-alpha-induced decrease of TER was not affected both in the HTNV-infected and uninfected cell monolayers (Fig. 4C). These results indicate that either NO or PG is not involved in the longer duration of increased permeability in the HTNV-infected monolayers. In fact, neither of them was involved in the TNF-alpha-induced increase in permeability in the HTNV-infected and uninfected monolayers.

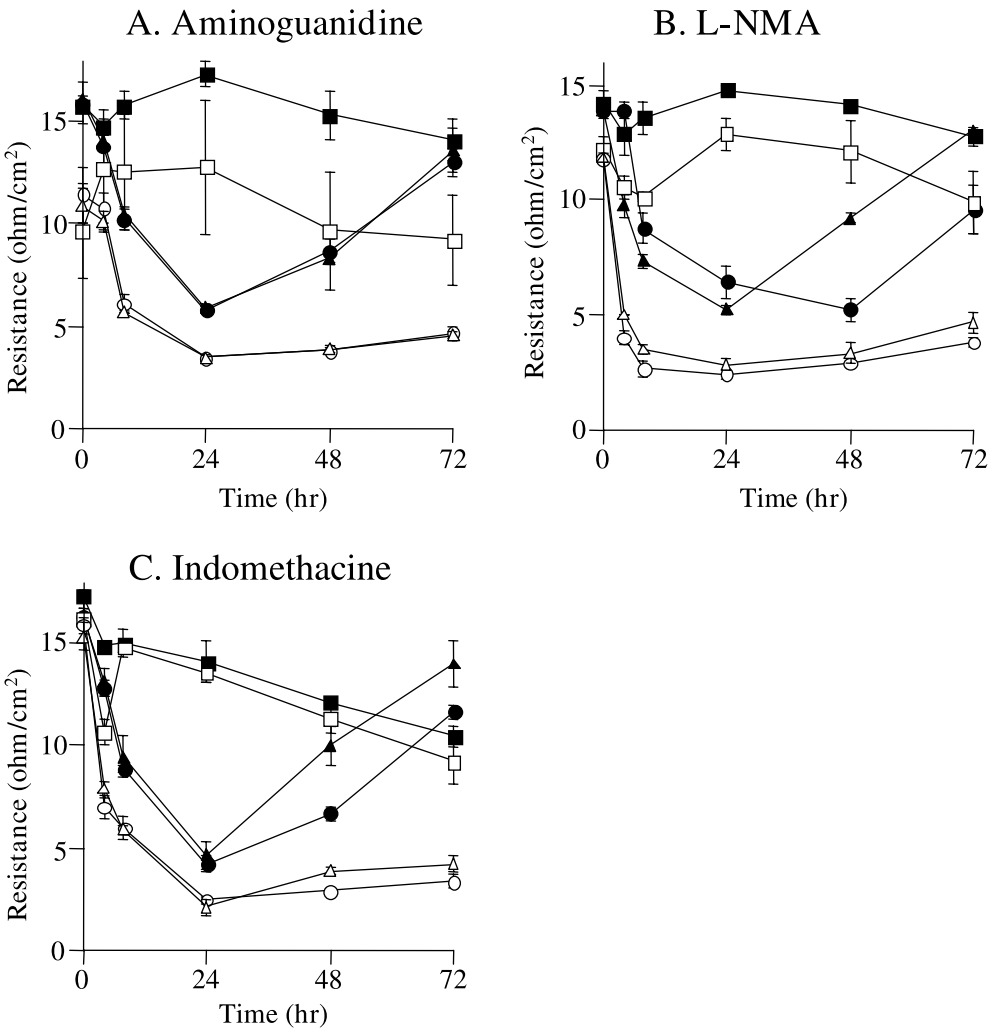

Fig. 4. Absence of the effect of NOS inhibitors and a cyclooxygenase inhibitor on TER. Aminoguanidine (100 μM, iNOS inhibitor, **A**), L-NMA (1 mM, eNOS inhibitor, **B**) or Indomethacin (30 μM, cyclooxygenase inhibitor, **C**) was added along with 1 ng/ml TNF-alpha and the TER was measured at 4, 8, 24, 48 and 72 hr after the stimulation. □, infected control HUVEC; ○, infected TNF-alpha-stimulated HUVEC; △, infected TNF-alpha-stimulated HUVEC with the inhibitor; ■, uninfected control HUVEC; ●, uninfected TNF-alpha-stimulated HUVEC; ▲, uninfected TNF-alpha-stimulated HUVEC with the inhibitor. Each point represents the mean value of triplicate. Bars indicate SD

Induction of pro-inflammatory cytokines by TNF-alpha stimulation

We next asked if the TNF-alpha-induced pro-inflammatory cytokines from endothelial cells might affect the permeability by autocrinal mechanisms. As shown in Fig. 5A, IL-6 was induced at significantly higher levels in the HTNV-infected HUVEC cultures than in the uninfected ones. In the HTNV-infected cells, IL-6 levels increased sharply during the first 24 hr after the stimulation and reached

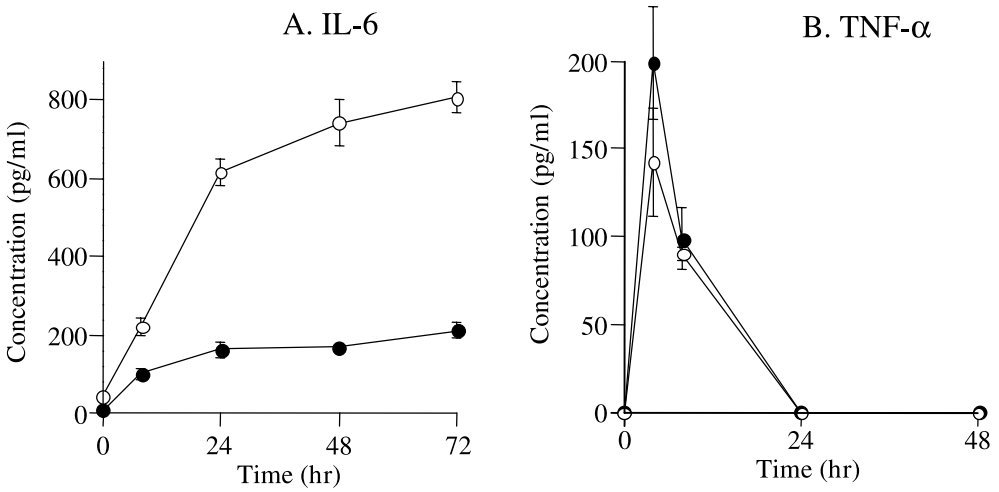

Fig. 5. Induction of high levels of IL-6 and absence of TNF-alpha induction by TNF-alpha. IL-6 (**A**) and TNF-alpha (**B**) concentrations in the supernatants of HTNV-infected (○) and uninfected (●) HUVEC were assayed at indicated hours after the addition of TNF-alpha (1 ng/ml). Time 0 indicates the concentrations before the addition of TNF-alpha. Each point represents the mean value of triplicate. Bars indicate SD

616 pg/ml. The concentration gradually increased further up to 807 pg/ml until 72 hr after the stimulation. On the other hand, in the uninfected cells, the concentration of IL-6 was slowly increased up to 163 pg/ml for the first 24 hr. During the next 48 hr, the concentration slightly increased and reached 210 pg/ml at 72 hr after the stimulation.

The HTNV infection of HUVEC itself did not induce TNF-alpha production (time 0 in Fig. 5B). The TNF-alpha concentration in the supernatants decreased rapidly after the addition of TNF-alpha, probably due to the degradation or absorbance to the cells, and completely disappeared at 24 hr after the addition. It was not detected thereafter until 96 hr (Fig. 5B and data not shown). At 4 hr after the stimulation, the concentration of TNF-alpha was marginally higher in the uninfected cell supernatant; however, the difference completely disappeared at 8 hr. Thus, the TNF-alpha stimulation did not induce significant level of TNF-alpha in either the HTNV-infected or uninfected HUVECs. IL-1beta was not produced above the detectable levels (10 pg/ml) by the TNF-alpha stimulation either in the HTNV-infected or uninfected HUVECs (data not shown).

We then investigated if the increased level of IL-6 was involved in the extension of hyper-permeability in HTNV-infected monolayers by adding the neutralizing antibody to IL-6 at the time of TNF-alpha stimulation. The antibody at concentrations of 50 or 500 ng/ml did not affect the time courses of hyper-permeability in either the HTNV-infected or uninfected monolayers (data not shown). Further, the uninfected monolayers treated with the mixtures of TNF-alpha and IL-6 did not show prolonged hyper-permeability (data not shown). These results suggest that IL-6 is not involved in the prolonged response of the HTNV-infected monolayers to TNF-alpha.

Discussion

We demonstrated that HTNV infection of HUVEC extended the duration of increased permeability of monolayers induced by low levels of TNF-alpha, while the magnitude of initial increase in permeability was comparable to the uninfected cells. In fact, this phenomenon was observed when 1 ng/ml of TNF-alpha was added to the culture medium, which is 10 times higher concentration than that detected in HFRS patients' sera [22]. This concentration is, at the same time, comparable to the reported concentration secreted by SNV-infected macrophages *in vitro* [17]. This is the first report demonstrating that the infection of hantavirus to endothelial cell monolayers modifies their barrier function. We speculate that the prolonged hyper-permeability of HTNV-infected endothelium induced by the TNF-alpha secreted from the infected monocytic cells may result in a gradual accumulation of hyper-permeable vascular endothelium (Fig. 6). In this hypothetical model, we assume the number of infected monocytic cells and the overall

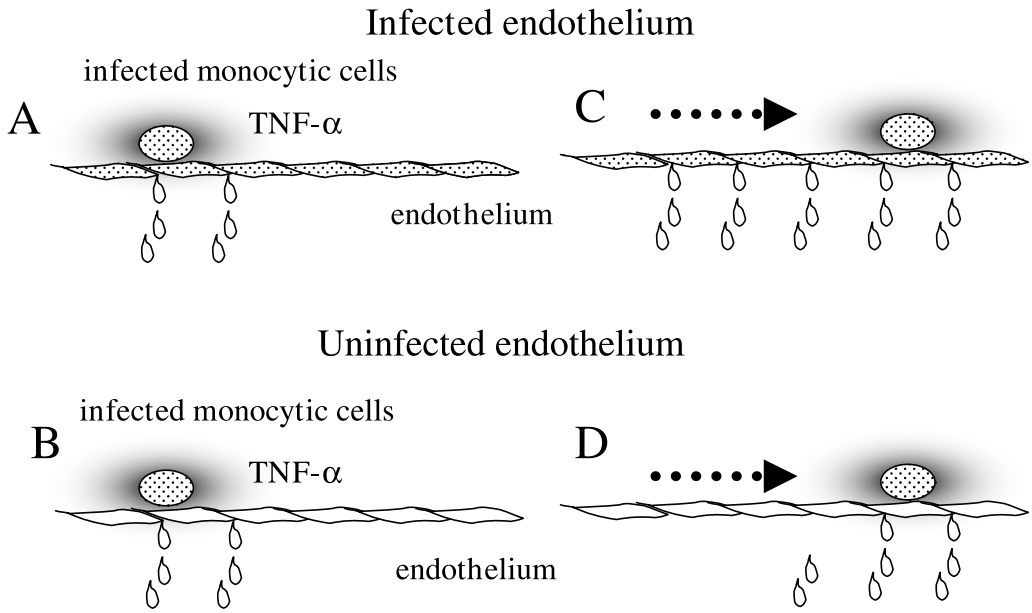

Fig. 6. A hypothetical scheme how the combination of the prolonged hyper-permeability of infected endothelium and the low-level TNF-alpha secretion from infected monocytic cells may contribute to a serious plasma leakage. In both infected and uninfected endothelium, the low-level TNF-alpha from the infected monocytic cells induces hyper-permeability only to the close proximity (**A** and **B**). When the infected monocytic cells move, plasma discharge continues from the infected endothelium which is no longer close proximity to the infected monocytic cells, as well as the leakage from the endothelium now becomes close proximity to them (**C**). Contrary, in the uninfected endothelium, only the endothelium newly becomes close proximity to the infected monocytic cells begins to leak, while the previously hyper-permeable endothelium recovers when the concentration of TNF-alpha is not high enough any more (**D**)

amount of secreted TNF-alpha from these cells are not great enough to induce systemic catastrophic change. On the other hand, the secretion of TNF-alpha from individual infected monocytic cell is high enough to induce prolonged hyper-permeability in the proximal infected endothelium. This might contribute to a local catastrophic plasma leakage after a certain period of time in specific organs where the infected endothelial cells and monocytic cells are close proximity. It should be noted that although HFRS is an acute disease, it takes order of weeks after infection before the critical condition appears [19]. A recent report showed that HTNV infection also induced dendritic cells to produce a low level of TNF-alpha [39]. Thus, the HTNV-infected monocytic cells can be a source of TNF-alpha *in vivo*, though TNF-alpha was supplied exogenously in our *in vitro* experiments. Of further interest, increased numbers of TNF-alpha producing cells in HPS patients' lungs were described and the involvement of local cytokine production in the HPS pathogenesis was suggested [32]. Furthermore, in dengue virus infection, which also causes hemorrhagic fever without serious damage on vascular endothelium, it was reported that TNF-alpha from the dengue virus-infected peripheral monocytes modulated endothelial cell protein expressions [1]. The mechanisms behind the hyper-permeability without disruption of the tight junction are not clear. One report suggested that the rearrangement of cytoskeleton by TNF-alpha changed the tension within the individual cells and resulted in hyper-permeability in endothelial monolayers without gap formation [2].

In HFRS and HPS patients' plasma, increased levels of NO were reported [9, 24]. In the former, the increased NO levels correlated to the TNF-alpha levels. However, our results suggested that NO was not involved in the prolonged hyper-permeability. The irrelevance of NO to the formation of pulmonary edema and alveolar flooding in a mice model system with lymphocytic choriomeningitis virus, which might mimic HPS, was recently reported [9]. The involvement of PG in the prolonged hyper-permeability was also not likely, while Cox-2 was one of the up-regulated genes by the HTNV infection in endothelial cells detected by the DNA array experiments [13].

The low level of TNF-alpha induced IL-6 above 700 pg/ml in the HTNV-infected HUVECs. This phenomenon might partly contribute to the elevated levels of IL-6 in HTNV-infected patients. It is noteworthy that a previous report showed no difference in IL-6 levels between the HTNV-infected and uninfected endothelial cells *in vitro*, as the pre-stimulation levels in our experiments [36]. The involvement of induced IL-6 in the pathogenesis of HTNV infection is not clear, though our data indicates that it is not involved in the prolonged hyper-permeability. In one report, IL-6 alone could induce hyper-permeability at higher concentrations above 20 ng/ml using normal bovine endothelial cell monolayers derived from carotid aorta [29]. In our study, HUVEC monolayers did not respond to IL-6 alone at concentrations up to 100 ng/ml (data not shown). Since endothelial cells derived from different organs or species may show different property [2, 26], it is not known whether this is due to the difference of source organs of the endothelial cells or to the species difference.

Difference in the sensitivity to cytokine stimulations in different species may even play a role in the pathogenesis of hantavirus infections. Hantaviruses are pathogenic to humans but not to the natural hosts, rodents, except for suckling animals [30, 34, 38, 44]. It is, therefore, tempting to postulate that the high sensitivity of human to pro-inflammatory cytokines [10] may be one of the possible explanations for the different pathogenesis between human and other animals, assuming that the combination of the low level of TNF-alpha and the endothelial cell infection plays a major role in the pathogenesis of human hantavirus infections.

Acknowledgements

We thank Ms. M. Ogata for her assistance and Dr. J. Arikawa for helpful discussion and providing HTNV. This study was supported by grants from the Ministry of Health, Welfare, and Labor and the Ministry of Education, Science, Culture and Sports of Japan.

References

1. Anderson R, Wang S, Osiowy C, Issekutz AC (1997) Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. J Virol 71: 4226–4232
2. Blum MS, Toninelli E, Anderson JM, Balda MS, Zhou J, O'Donnell L, Pardi R, Bender JR (1997) Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. Am J Physiol 273: H286–H294
3. Bonner SM, O'Sullivan MA (1998) Endothelial cell monolayers as a model system to investigate dengue shock syndrome. J Virol Methods 71: 159–167
4. Bove K, Neumann P, Gertzberg N, Johnson A (2001) Role of ecNOS-derived NO in mediating TNF-induced endothelial barrier dysfunction. Am J Physiol-Lung Cell Mol Physiol 280: L914–L922
5. 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
6. Butler JC, Peters CJ (1994) Hantaviruses and hantavirus pulmonary syndrome. Clin Infect Dis 19: 387–394
7. Cosgriff TM (1991) Mechanisms of disease in hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome. Rev Infect Dis 13: 97–107
8. De Assis MC, Da Casta AO, Barja-Fidalgo TC, Plotkowski MC (2000) Human endothelial cells are activated by interferon-gamma plus tumor necrosis factor-alpha to kill intracellular *Pseudomonas aeroginosa.* Immunology 101: 271–278
9. Davis IC, Zajac AJ, Nolte KB, Botten J, Hjelle B, Matalon S (2002) Elevated generation of reactive oxygen/nitrogen species in hantavirus cardiopulmonary syndrome. J Virol 76: 8347–8359
10. Dinarello CA (1996) Cytokines as mediators in the pathogenesis of septic shock. Curr Topic Microbiol Immunol 216: 133–165
11. Fisher-Hoch SP, Platt GS, Neild GH, Southee T, Baskerville A, Raymond G, Lloyd RT, Simpson DI (1985) Pathophysiology of shock and hemorrhage in a fulminating viral infection (Ebola). J Infect Dis 152: 887–894
12. Freyer D, Manz R, Ziegenhorn A, Weih M, Angstwurm K, Docke WD, Meisel A, Schumann RR, Schonfelder G, Dirnagl U, Weber JR (1999) Cerebral endothelial cells release TNF-alpha after stimulation with cell walls of *Streptococcus pneumoniae* and

regulate inducible nitric oxide synthase and ICAM-1 expression via autocrine loops. J Immunol 163: 4308–4314
13. Geimonen E, Neff S, Raymond T, Kocer SS, Gavrilovskaya IN, Mackow ER (2002) Pathogenic and nonpathogenic hantavuris differently regulate endothelial cell responses. Proc Natl Acad Sci USA 99: 13837–13842
14. Goldblum SE, Sun WL (1990) Tumor necrosis factor-alpha augments pulmonary arterial transendothelial albumin flux in vitro. Am J Physiol 258: L57–L67
15. Griffith OW, Kilbourn RG (1996) Nitric oxide synthase inhibitors: Amino acids. In: Packer L (ed) Nitric oxide part A. Methods in enzymology 268. Academic Press, New York, pp. 375–392
16. Kang JI, Park SH, Lee PW, Ahn BY (1999) Apoptosis is induced by hantaviruses in cultured cells. Virology 264: 99–105
17. Khaiboullina SF, Netski DM, Krumpe P, St Jeor SC (2000) Effects of tumor necrosis factor alpha on Sin Nombre virus infection *in vitro*. J Virol 74: 11966–11971
18. Kim GR, McKee KT Jr (1985) Pathogenesis of Hantaan virus infection in suckling mice: clinical, virologic, and serologic observations. Am J Trop Med Hyg 34: 388–395
19. Klingstrom J, Plyusnin A, Vaheri A, Lundkvist A (2002) Wild-type Puumala hantavirus infection induces cytokines, C-reactive protein, creatinine, and nitric oxide in cynomolgus macaques. J Virol 76: 444–449
20. Lampugnani MG, Corada M, Caveda L, Breviario F, Ayalon O, Geiger B, Dejana E (1995) The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). J Cell Biol 129: 203–217
21. Linderholm M, Billstrom A, Settergren B, Tarnvik A (1992) Pulmonary involvement in nephropathia epidemica as demonstrated by computed tomography. Infection 20: 263–266
22. Linderholm M, Ahlm C, Settergren B, Waage A, Tarnvik A (1996) Elevated plasma levels of tumor necrosis factor (TNF)-alpha, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients with hemorrhagic fever with renal syndrome. J Infect Dis 173: 38–43
23. Linderholm M, Groeneveld PH, Tarnvik A (1996) Increased production of nitric oxide in patients with hemorrhagic fever with renal syndrome – relation to arterial hypotension and tumor necrosis factor. Infection 24: 337–340.
24. Lukes RJ (1954) The pathology of thirty-nine fatal cases of epidemic hemorrhagic fever. Am J Pathol 16: 639–650
25. Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W (1993) Tumor necrosis factor α (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor type, TNF-R55. J Exp Med 177: 1277–1286
26. Mantovani A, Bussolino F, Dejana, E (1992) Cytokine regulation of endothelial cell function. FASEB J 6: 2591–2599
27. Mantovani A, Bussolino F, Introna M (1997) Cytokine regulation of endothelial cell function: from molecular level to the bedside. Immunol Today 18: 231–240
28. Mark KS, Trickler WJ, Miller DW (2001) Tumor necrosis factor-alpha induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. J Pharmacol Exp Ther 297: 1051–1058
29. Maruo N, Morita I, Shirao M, Murota S (1992) IL-6 increases endothelial permeability in vitro. Endocrinology 131: 710–714
30. McKee KT Jr, Kim GR, Green DE, Peters CJ (1985) Hantaan virus infection in suckling mice: virologic and pathologic correlates. J Med Virol 17: 107–117

31. Meyer BJ, Schmaljohn CS (2000) Persistent hantavirus infections: characteristics and mechanisms. Trend Microbiol 8: 61–67
32. Mori M, Rothman AL, Kurane I, Montoya JM, Nolte KB, Norman JE, Waite DC, Koster FT, Ennis FA (1999) High levels of cytokine-producing cells in the lung tissues of patients with fatal hantavirus pulmonary syndrome. J Infect Dis 179: 295–302
33. Munro JM, Pober JS, Cotran RS (1989) Tumor necrosis factor and interferon-gamma induce distinct patterns of endothelial activation and associated leukocyte accumulation in skin of Papio anubis. Am J Pathol 135: 121–133
34. Nakamura T, Yanagihara R, Gibbs CJ Jr, Amyx HL, Gajdusek DC (1985) Differential susceptibility and resistance of immunocompetent and immunodeficient mice to fatal Hantaan virus infection. Arch Virol 86: 109–120
35. Nolte KB, Feddersen RM, Foucar K, Zaki SR, Koster FT, Madar D, Merlin TL, McFeeley PJ, Umland ET, Zumwalt RE (1995) Hantavirus pulmonary syndrome in the United States: a pathological description of a disease caused by a new agent. Human Pathol 26: 110–120
36. Pensiero MN, Sharefkin JB, Dieffenbach CW, Hay J (1992) Hantaan virus infection of human endothelial cells. J Virol 66: 5929–5936
37. Peters CJ, Khan AS (2002) Hantavirus pulmonary syndrome: The New American hemorrhagic fever. Clin Infect Dis 34: 1224–1231
38. Plyusnin A, Vapalahti O, Vaheri A (1996) Hantaviruses: genome structure, expression and evolution. J Gen Virol 77: 2677–2687
39. Raftery MJ, Kraus AA, Ulrich R, Kruger DH, Schonrich G (2002) Hantavirus infection of dendritic cells. J Virol 76: 10724–10733
40. Royall JA, Berkow RL, Beckman JS, Cunningham MK, Matalon S, Freeman BA (1989) Tumor necrosis factor and interleukin 1 alpha increase vascular endothelial permeability. Am J Physiol-Lung Cell Mol Physiol 257: L399–L410
41. Sugiyama K, Morikawa S, Matsuura Y, Tkachenko EA, Morita C, Komatsu T, Akao Y, Kitamura T (1987) Four serotypes of haemorrhagic fever with renal syndrome viruses identified by polyclonal and monoclonal antibodies. J Gen Virol 68: 979–987
42. Sundstrom JB, McMullan LK, Spiropoulou CF, Hooper WC, Ansari AA, Peters CJ, Rollin PE (2001) Hantavirus infection induces the expression of RANTES and IP-10 without causing increased permeability in human lung microvascular endothelial cells. J Virol 75: 6070–6085.
43. Yanagihara R, Silverman DJ (1990) Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses. Arch Virol 111: 281–286
44. Yoshimatsu K, Arikawa J, Ohbora S, Itakura C (1997) Hantavirus infection in SCID mice. J Vet Med Sci 59: 863–868
45. Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, Feddersen RM, Zumwalt RE, Miller GL, Khan AS, Rollin PE, Ksiazek TG, Nicole ST, Mahy BWJ, Peters CJ (1995) Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease. Am J Pathol 146: 552-579

Author's address: Shigeru Morikawa, DVM, Ph.D., 4-7-1 Gakuen, Musashimurayam, 208–0011 Tokyo, Japan; e-mail: morikawa@nih.go.jp