JOURNAL OF VIROLOGY, Oct. 1992, p. 5929–5936
0022-538X/92/105929-08$02.00/0

Vol. 66, No. 10

Hantaan Virus Infection of Human Endothelial Cells

MICHAEL N. PENSIERO,[1]†* JOHN B. SHAREFKIN,[2] CARL W. DIEFFENBACH,[3] AND JOHN HAY[1]‡

Departments of Microbiology[1] and Pathology,[3] Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799, and Vascular Research Group, New England Medical Center Hospitals, Boston, Massachusetts 02111[2]

Received 28 February 1992/Accepted 8 July 1992

The primary pathophysiologic finding of the viral disease known as Korean hemorrhagic fever, the etiological agent of which is Hantaan virus (HTV), is vascular instability. To investigate whether HTV was able to infect cells derived from human vascular tissue and alter their behavior, we infected in vitro primary adult human endothelial cells from saphenous veins (HSVEC). We were able to detect the presence of viral antigens in infected cells both by immunofluorescence and by Western blot (immunoblot) analysis as early as day 1 postinfection. HSVEC infected with HTV produce infectious virus during the first 3 days of infection but, at later times (days 4 to 8), show decreasing yields of virus. This contrasts with the HTV growth pattern observed for the permissive simian CV-7 cell line, which generates infectious virus up to day 12 after infection. Further investigation showed that the late decrease in viral production in HSVEC is the result of the induction of beta interferon and can be reversed by the addition of anti-beta interferon serum to the culture medium. At no time during the course of infection of HSVEC with HTV was any obvious cytopathic effect observed. When tests for changes in mRNA levels of other cytokines and endothelial cell gene products following HTV infection of HSVEC were done by reverse transcription and polymerase chain reaction methods, no significant changes were observed in the levels of interleukin 1, interleukin 6, or von Willebrand factor mRNA. We hypothesize that, while HTV can replicate in human vascular endothelial cells, the mechanism of microvascular damage seen with Korean hemorrhagic fever is not likely to be a direct effect of virus replication but may conceivably be the consequence of an immune-mediated endothelial injury triggered by viral infection.

Hemorrhagic fever with renal syndrome (HFRS) is caused by a group of serologically related viruses belonging to the hantavirus genus of the family *Bunyaviridae*. Hantaan virus (HTV), the etiologic agent of Korean hemorrhagic fever, is the prototype virus of this family (26). In 1976, HTV was isolated from the lungs of the Korean striped field mouse, *Apodemus agrarius*. Adaptation of HTV to cell culture and the development of an immunofluorescence assay have allowed serological diagnosis of HTV and HTV-related infections (16). As a result, seroepidemiological studies have demonstrated that hantaviruses are widespread throughout the world in several genera of rodents (15). These viruses are maintained in nature through asymptomatic infections, and it is believed that humans become infected through contact with aerosols of rodent excreta. At least five distinct serotypes of hantaviruses have been identified: HTV, Seoul, Puumala, Prospect Hill, and Thottlapalyam (2, 16). The spectrum of illness caused by hantaviruses varies with the particular virus involved: HTV and Seoul are associated with severe and moderate forms of HFRS, Puumala is associated with a mild form of HRFS, and Prospect Hill, isolated from *Microtus pennsylvanicus*, is a nonpathogenic hantavirus.

The principal pathophysiologic finding in HFRS is an increase in vascular permeability, commonly affecting systemic small blood vessels and capillaries (6). At present, there is no consensus on the mechanism behind the disease, but viral antigens have been demonstrated in the vascular endothelia of tissues from suckling mice experimentally infected with HTV as well as in the endothelia of autopsy specimens from patients with HFRS (12, 14). An understanding of the pathogenesis of HFRS would be aided by development of an in vitro system for the study of HTV infection of human vascular endothelial cells. Two recent investigations have shown that HTV antigens can be detected in endothelial cells from human umbilical vein cells (HUVEC) after inoculation with HTV (22, 33). However, it is not clear from either of these studies whether the cells can support a productive infection by HTV or what the characteristics of the virus-cell relationship might be. To develop a defined system for the interaction between HTV and human endothelial tissue, we have extended these earlier studies using HTV infection of primary adult human endothelial cells isolated from saphenous veins (HSVEC). Our results show that HTV infection of HSVEC or HUVEC leads to a limited productive growth pattern compared with the growth pattern seen with the permissive CV-7 cell line. We demonstrate that the growth of HTV is limited in vascular cells by the induction of beta interferon (IFN-β). At no time during the course of HTV infection was any cytopathic effect observed in vascular endothelial cells. Furthermore, the polymerase chain reaction (PCR) analysis of mRNA levels for other endothelial cell gene products following HTV infection demonstrated no significant change in the steady-state mRNA levels of interleukin 1 (IL-1), IL-6, or von Willebrand factor (vWF).

MATERIALS AND METHODS

Cells, virus, and antisera. The 76-118 strain of HTV, originally isolated from *A. agrarius*, was obtained from the American Type Culture Collection (Rockville, Md.); all manipulations were conducted under BL-3 containment.

* Corresponding author.
† Present address: Genetic Therapy, Inc., 19 Firstfield Road, Gaithersburg, MD 20878.
‡ Present address: Department of Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, NY 14214.

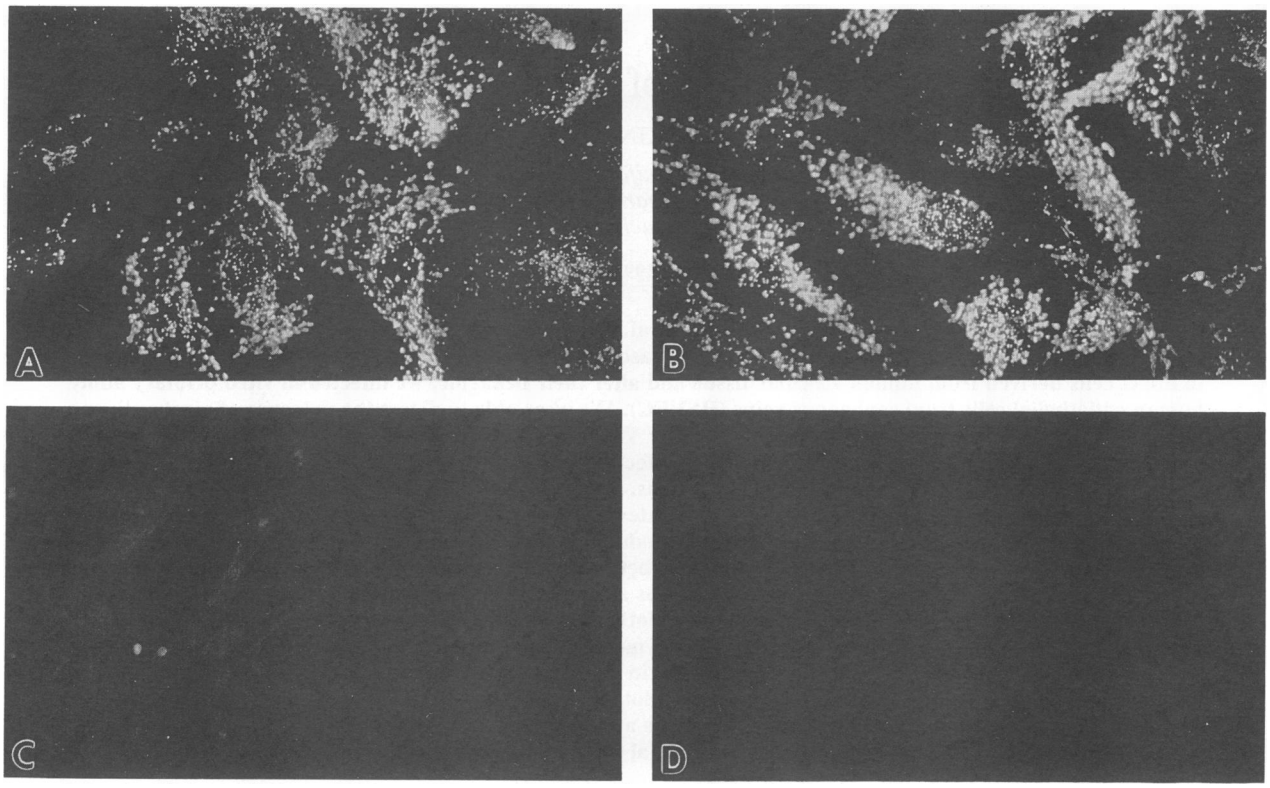

FIG. 1. Staining of primary human cells for vWF. (A and B) HSVEC (passage 3); (C) WI38 cells (human diploid lung fibroblasts); (D) human foreskin fibroblasts. The antiserum used in immunofluorescence was a mouse monoclonal antibody to human vWF, and the secondary antibody was a rhodamine-conjugated goat anti-mouse serum. The rod-shaped fluorescence seen in panels A and B is typical of Wiebel-Palade bodies contained in endothelial cells. Magnification, ×630.

HTV was propagated in VBCV-7 cells, a clone derived from CV-1 cells, which were the kind gift of Sherman Hasty (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.). This particular clone was shown to be particularly effective in the growth of HTV (12a). Titers of HTV were determined by plaque assay on CV-7 cells as previously described (8). HCO2, a monoclonal antibody directed against the HTV G2 glycoprotein, and polyclonal rabbit anti-HTV serum, both kindly donated by Connie Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases), were used as previously described (27). Sheep antiserum to human IFN-β (catalog no. G-028-501-568) was obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases (Bethesda, Md.). Monoclonal antibody to human vWF (anti-vWF) was obtained commercially from Boehringer Mannheim (catalog no. 1284-924). Immunofluorescence was performed by acetone fixation of infected cells grown on glass coverslips as previously described (21).

Harvest and culture of HSVEC. Saphenous vein segments normally discarded during coronary bypass operations were obtained under protocols approved by the human study review committees of our institutions. Vein segments were transported from the operating room on ice in heparinized whole blood. Vein segments were cannulated at their distal ends, flushed free of blood with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), filled with a sterile solution of 0.1% class II-type collagenase (Worthington Biochemical, Freehold, N.J.) and 0.5% bovine serum albumin, and clamped with a hemostat at their proximal ends to retain the enzyme solution and maintain slight distension. Veins were incubated in sterile Hanks balanced salt solution at 37°C for 15 to 20 min. The dissociated endothelial cells and collagenase solution were then flushed out with 10 ml of medium 199 containing 100 μg of porcine heparin (type H3125; Sigma) per ml, 100 mg of L-glutamine per liter, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 2.5 μg of amphotericin B (Fungizone) per ml, and 10% fetal calf serum (Hyclone serum; Sterile Systems, Logan, Utah). The flushed cell suspension was centrifuged at 250 × *g* at 4°C for 5 min, and the cell pellet was resuspended in medium 199 with 20% serum and the same supplements as above plus 5 μl of a saline extract of bovine retina containing heparin-binding endothelial cell growth factors per ml, as previously described (28). Cells were plated in gelatin-coated T-25 flasks at initial densities of approximately 3,000 to 10,000 cells per cm^2 and grown at 37°C in a 5% CO_2 atmosphere until confluent. Confluent cultures were passaged at 1:3 split ratios by using a solution of 0.02% EDTA–0.005% crystalline trypsin (VMF Trypsin, Worthington, Del.) and studied in passages 2 through 5. The endothelial identity of the cells was verified by a homogeneous cobblestone monolayer morphology on phase-contrast microscopic examination, positive immunofluorescent staining for vWF (Fig. 1), and electron microscopic confirmation of the presence of Wiebel-Palade bodies (10).

Primary cultures of HUVEC were obtained as previously described. HUVEC were passaged and maintained as described above (31).

Isolation of total RNA from HTV-infected endothelial cell

monolayers. Endothelial cells grown in 100-mm gelatin-coated tissue culture dishes were infected with HTV at a multiplicity of infection of 0.1. At designated times postinfection, monolayers were washed three times in cold PBS, and then 3 ml of RNAzol B (Teltest Laboratories International, Friendswood, Tex.) was added. The cell lysate was then transferred to a Corex tube to which 0.2 ml of chloroform was then added. Samples were vigorously vortexed for 15 min and then placed on ice for 5 min. The suspension was spun at 12,000 × *g* (4°C) for 15 min, the upper aqueous layer was transferred to a new Corex tube, and 3 ml of isopropanol was added. The sample was spun for 15 min at 12,000 × *g* (4°C), after which the supernatant was decanted. The RNA pellet was then washed in 70% ethanol, repelleted, resuspended in 300 μl of doubly distilled H_2O, and heated to 65°C for 10 min. Then, one-fourth volume of 10 M $LiCl_2$ was added to the mixture. Samples were then placed on ice for 3 h, after which the RNA was repelleted and washed two times in 70% ethanol. RNA pellets were dried and resuspended in 100 to 200 μl of H_2O to give a final concentration of 1 μg/μl.

Reverse transcription and PCR. The RNA PCR was performed as described by Dveksler et al. (9). Briefly, 10 μg of RNA was incubated with 2 U of RQ DNase (Promega Corporation, Madison, Wis.) for 15 min at room temperature, and then the RNA was extracted with 50:50 phenol-chloroform. Reverse transcription reactions were carried out in a volume of 20 μl containing 2.0 μg of total RNA, 500 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.), 1 μg of random hexanucleotide primers, and 10 mM deoxynucleoside triphosphates. Each reverse transcription reaction was carried out for 20 min at 37°C and then stopped by denaturation for 10 min at 90°C.

The resulting cDNA samples were amplified by using 20 cycles of the PCR with sense and antisense primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (9), human IFN-β (13), human IL-1α and IL-1β (11), IL-6 (11), the HTV M segment (32), generated from previously published sequences. Human alpha interferon (IFN-αA/D) primers were used as modified by an earlier procedure (11). Sense and antisense primers for detection of vWF mRNA were derived from exons 21 (sense primer) and 23 (antisense primer) by using the vWF sequence published by Mancuso et al. (18) and by Bonthron et al. (3). The reactions were performed as follows: the 100-μl PCR volume contained 2 U of recombinant *Thermus aquaticus* (*Taq*) polymerase (Perkin Elmer-Cetus, Norwalk, Conn.); 10 mM each dCTP, dATP, dGTP, and dTTP (Pharmacia Inc., Piscataway, N.J.); and 400 ng each of sense and antisense primers in a reaction buffer of 50 mM KCl–100 mM Tris-HCl (pH 8.3)–25 mM $MgCl_2$ in 1% (wt/vol) gelatin. Each PCR cycle consisted of 1.5 min of denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min of primer extension at 72°C (Perkin Elmer-Cetus 1000 Thermocycler).

From each reaction, 10 μl of PCR product was electrophoresed on a 2% Seakem GTG agarose gel (FMC Corp., Rockland, Maine) in the presence of a 123-bp ladder (Bethesda Research Laboratories) used as a DNA standard. The gels were stained with ethidium bromide, photographed, washed, and then blotted overnight to nylon membranes with a 0.45-μm pore size (Nytran; Schleicher & Schuell Inc., Keene, N.H.). Southern blots of the gels were probed for GAPDH, IFN-α, IFN-β, and the HTV M segment by using internal oligonucleotides end-labelled with ^{32}P by incubation with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase. Autoradiographs were exposed at −70°C with Kodak XAR film. Quantitation was effected by exposing blots to a phosphor screen and subsequent scanning in a PhosphorImager system (Molecular Dynamics). Data were analyzed and quantitated by using ImageQuant software (Molecular Dynamics). Levels of particular gene products were normalized to GAPDH levels.

The oligonucleotides used in the PCR experiments were as follows. For IL-1β, the primers were AAAAGCTTGGTG ATGTTCTGG (sense) and TTTCAACACGCAGGACAGG (antisense), and the probe was ATGGAGCAACAAGTGG TG; the product was 187 bp. For IL-1α, the primers were TTCGAGCCAATGATCAGTACC (sense) and TTTGGGTA TCTCAGGCATCTCC (antisense), and the probe was GAT GAAGCAGTGAAATTTGAC; the product was 203 bp: for IL-6, the primers were GTGTGAAAGCTGCAAAGAGGC (sense) and CTGGAGGTACTCTAGGTATAC (antisense), and the probe was GGATTCAATGAGGAGACTTGC; the product was 159 bp. For IFN-α, the primers were GGA AGCCTGTGTGTGATACAG (sense) and GATCTCATGA TTTCTGCTCTGA (antisense), and the probe was GGCTG TGAGGAAATACTTCCA; the product was 214 bp. For IFN-β, the primers were GATTCATCTAGCACTGGCTG (sense) and CTTCAGGTAATGCAGAATCC (antisense), and the probe was GAGAACCTCCTGGCTAATGTC; the product was 166 bp. For vWF, the primers were GATC CTAGTGGGGAATAAGG (sense) and TGGTGAGGTCA TTGTTCTGG (antisense), and the probe was CCCATGAA GGATGAGACTCACT; the product was 308 bp. For GA PDH, the primers were CCATGGAGAAGGCTGGG (sense) and CAAAGTTGTCATGGATGACC (antisense), and the probe was CTAAGCAGTTGGTGGTGCA; the product was 195 bp. For HTV G2, the primers were TGGAATGACAA TGCCCATGGG (sense) and ATGGATTACAACCCCAGC TCG (antisense), and the product was 334 bp.

HTV growth in the presence of anti-IFN-β antiserum. Duplicate wells of a 24-well plate containing CV-7 cells or HSVEC were either mock infected or infected with HTV at a multiplicity of infection of 0.1. After adsorption, cells were incubated with either 120 neutralizing units of sheep antiserum to human IFN-β or a control sheep antiserum. At 3 days postinfection, medium was removed from each well in duplicate, and the level of infectious virus was determined by a plaque assay. The remaining wells were reboosted with an additional 120 neutralizing units of sheep antiserum, and the infection was allowed to continue for 2 more days, after which the virus yield was again analyzed as described above.

RESULTS

Characterization of human endothelial cell cultures. When endothelial cells are prepared from the lining of a vein and are placed in culture, it is essential to verify that the culture is homogeneous for these cells. The reason for this is that the procedures used may also trap other cell types, such as smooth muscle or fibroblasts. The marker we use to identify endothelial cells is the presence of vWF (or factor VIII-related antigen), a plasma glycoprotein that mediates platelet adhesion to the subendothelium after vascular injury. In endothelial cells, vWF protein is concentrated in organelles called Weibel-Palade bodies and is not found in other cells, such as smooth muscle or fibroblasts. Figure 1 shows a variety of human cells stained with a monoclonal anti-human vWF antibody. Reaction is seen only for HSVEC (Fig. 1A and B), while human fetal lung cells (Fig. 1C) and human foreskin fibroblasts (Fig. 1D) show no staining. The positive

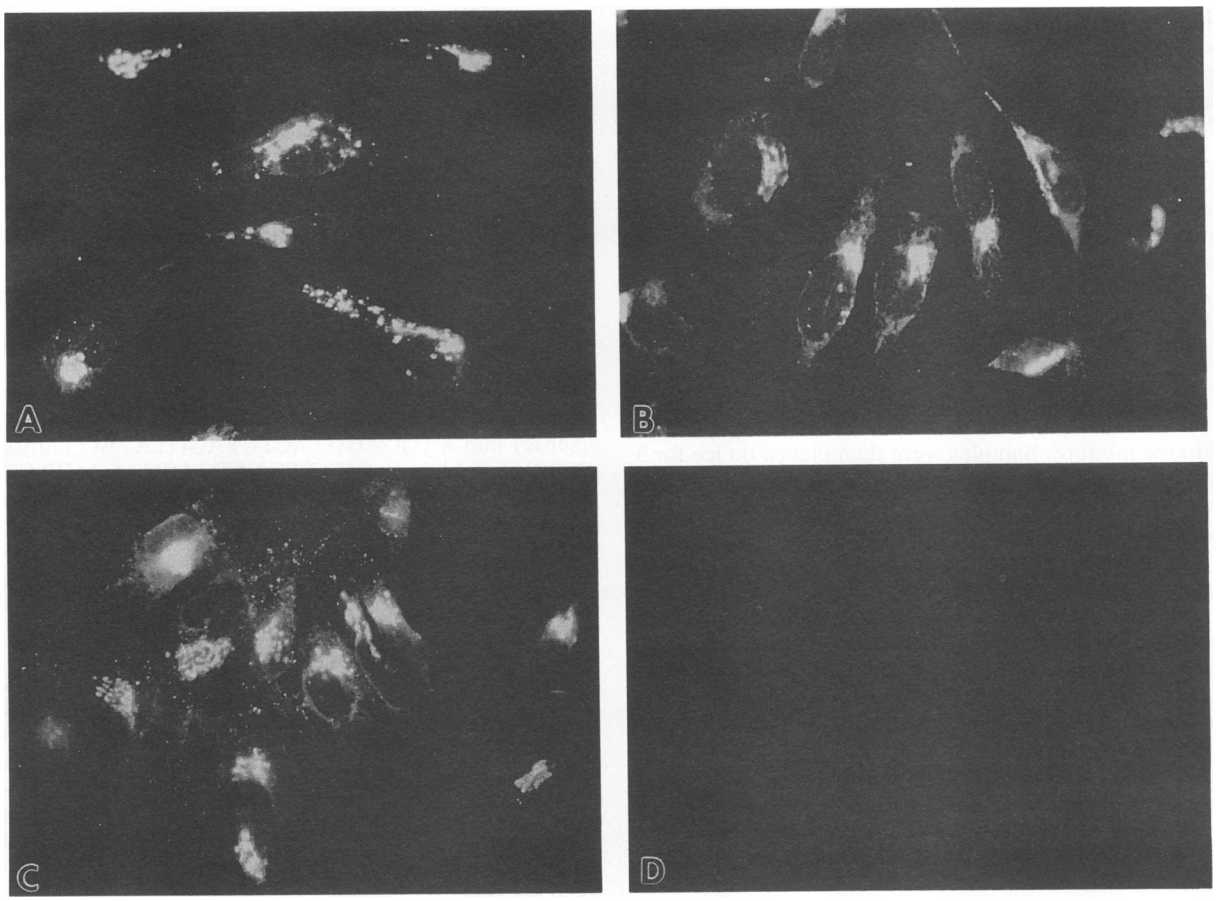

FIG. 2. Infection of HSVEC and HUVEC. Cells were infected with HTV at a multiplicity of infection of 0.1 and examined for viral antigens by immunofluorescence at 3 days postinfection. (A) HTV-infected HSVEC; (B) HTV-infected HUVEC; (C) HTV-infected CV-7 cells; (D) mock-infected HSVEC. The antiserum used was a monoclonal antibody directed against the HTV G2 glycoprotein. Magnification, ×630.

fluorescence pattern, typical of vWF, is the result of its concentration in rod-shaped Weibel-Palade bodies. On the basis of these analyses, the cultures we used in all subsequent experiments were shown to be homogeneous cultures of human endothelial cells. Both HSVEC and HUVEC preparations demonstrated analogous levels of purity (>99%) (data not shown).

Viral antigen and infectious virus in HTV-infected human endothelial cells. HSVEC, HUVEC, or CV-7 cells were infected with HTV or mock infected and examined for the presence of virus antigen. Microscopic analysis of HTV-infected endothelial cells did not, at any time, show a cytopathic effect, a situation similar to that for permissive simian cells. However, as shown in Fig. 2, a 3-day HTV infection results in the presence of viral antigens in adult (HSVEC) and fetal (HUVEC) endothelial cells, as well as in CV-7 cells. In addition, it was clear that all the endothelial cells produced HTV antigen by day 2, whereas only a fraction of the CV-7 population was positive at this early time (data not shown). The Golgi-like fluorescence characteristic of HTV-infected cells was obvious in all cell types (Fig. 2), and this character was retained in samples taken at all subsequent times. These results show that human endothelial cells can support HTV antigen production and suggest that the kinetics of HTV antigen production may be even more rapid in endothelial cells than in permissive CV-7 cells.

However, the presence of virus antigen in infected cells does not tell us whether the virus is actually multiplying. To address this question, medium was removed from HTV-infected endothelial or CV-7 cells at daily intervals after infection. The production of infectious virus in the medium was determined by plaque assay, and the results are shown in Fig. 3. HSVEC showed production of HTV on day 2, the level of which declined gradually over the next few days. CV-7 cells, on the other hand, continued to produce high levels of virus for the duration of the experiment (and beyond) (data not shown).

A recent report by Chen et al. (5) showed that Punta Toro virus (a phlebovirus) buds basolaterally from polarized cells. To test whether any HTV was "trapped" underneath endothelial cells or was cell associated in a more general sense, we also harvested infected cells and assayed them for virus. We were unable to detect any significant amounts of cell-associated virus from any of these cells (data not shown).

Though endothelial cells did not produce significant levels of infectious HTV after day 5, this observation is not likely to have been the result of cell death. Light microscopic observations of infected cells revealed no cytopathic effect, as noted above, and electron microscopic analysis of infected HSVEC on day 6 showed 99% viability on the basis of the morphological characteristics of viable endothelial cells. In addition, viral antigens are produced in endothelial cells,

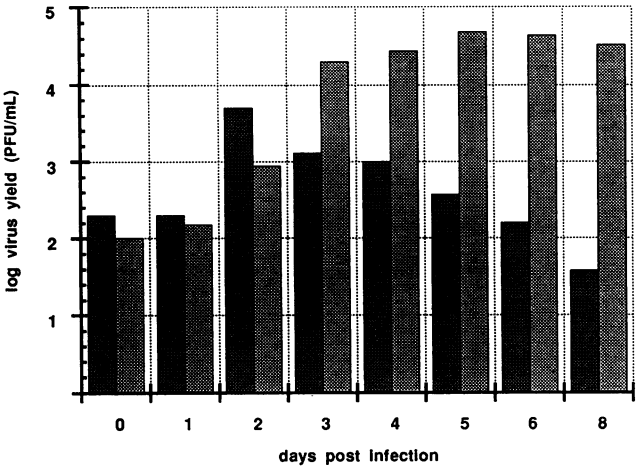

FIG. 3. Growth of HTV. CV-7 cells (▨) or HSVEC (■) were infected with HTV at a multiplicity of infection of 0.5, and at daily intervals postinfection, cell medium was removed and assayed for infectious virus by a plaque assay on CV-7 cells. Each bar on the graph represents an average of triplicate samples, with an average standard deviation of not more than 10% for each point.

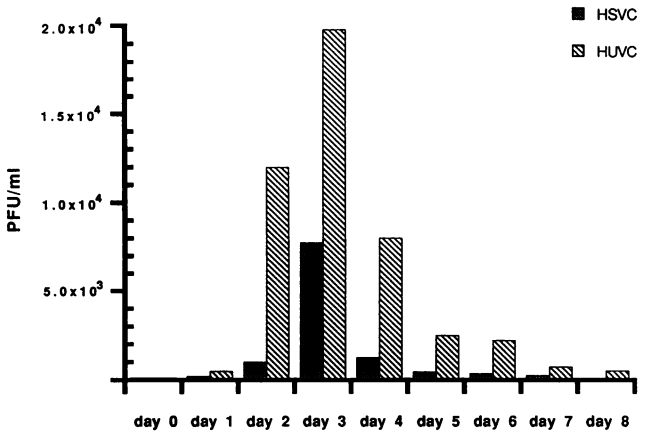

FIG. 5. Comparison of HTV growth in HSVEC and HUVEC. HUVEC and HSVEC were infected with HTV as described in the legend to Fig. 3. Amounts of infectious virus were determined from cell supernatants by a plaque assay on CV-7 cells. Bars on the graph represent the average of triplicate samples for each time point, with an average standard deviation of not more than 10% for each point.

even at quite late times after infection. Figure 4 shows the presence of the HTV nucleocapsid protein (N) in HSVEC at day 5, and 8-day-old HTV-infected endothelial cells show levels of N comparable to those of CV-7 cells (data not shown), even though no HTV virions could be seen in endothelial cells at these times (data not shown). We were unable to screen for viral glycoproteins in this assay, because there is no available antibody which functions in a Western blot (immunoblot).

To determine whether this HTV growth profile (Fig. 3) was specific for adult endothelial cells, we examined fetal endothelial cell cultures (HUVEC) after infection with HTV. Figure 5 shows the results of this experiment. Though the yields of infectious HTV are somewhat higher in HUVEC, it appears that the HTV growth pattern is generally similar to that in HSVEC. Both of these endothelial cell types are awkward to work with beyond approximately 10 days, making prolonged study of virus growth difficult.

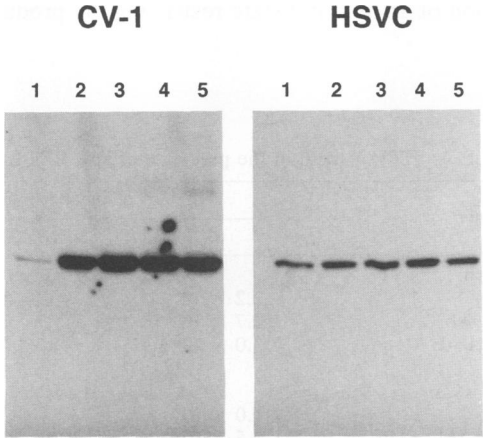

FIG. 4. The synthesis of HTV nucleocapsid protein in HSVEC. Cells infected with HTV as described in the legend to Fig. 3 were harvested for protein by lysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, assayed on polyacrylamide gels, blotted onto nitrocellulose, and probed with a rabbit polyclonal antiserum against HTV. Virus protein was detected by incubation with ^{125}I-staphylococcal protein A. Numbers above each lane represent the time (days) postinfection at which cells were harvested.

PCR analysis of cellular mRNA from HTV-infected endothelial cells. Since it is well documented that endothelial cells produce a variety of substances involved in immune responses, we chose to examine whether infection with HTV resulted in an alteration in the normal synthesis of these gene products. To test this, we used the PCR to measure changes in mRNA levels.

Since we have shown that our HSVEC cultures are homogeneous, we feel confident that any mRNA induction that we might detect is the result of HTV infection of a specific endothelial cell gene, as opposed to genes of a contaminating cell type. Specific PCR primers were made against the following endothelial cell genes: IL-1α, IL-1β, IL-6, vWF, IFN-α, and IFN-β. RNA was isolated from 1-day postinfection and 3-day postinfection HSVEC cultures either infected with HTV or mock infected and then subjected to PCR mRNA analysis. In these experiments, quantitation of mRNA was done on the basis of standardization, with the levels of cellular GAPDH mRNA amplified as an internal PCR control. This was chosen as an internal control since the level of this message in cells in culture is constant under a wide variety of growth conditions (9). Confirmation of a productive HTV infection was determined by the production of a 334-bp fragment by using HTV-specific PCR primers for the M segment.

As can be seen in Fig. 6, HTV infection of HSVEC had no significant effect on the levels of IL-6 or vWF mRNA. The ratios of counts per minute for HTV-infected cells to those for mock-infected cells usually varied from 0.8- to 1.4-fold. Immunofluorescence of HTV-infected cells stained with anti-vWF antibody also appeared similar to that of mock-infected cells in terms of absolute levels and distribution of vWF antigen (data not shown). We were unable to detect any signal above background from RNA isolated from mock-infected or HTV-infected HSVEC by using primers specific for IL-1α or IL-1β.

One of the likely reasons for the decreasing yields of HTV in endothelial cells at later times of infection might be the

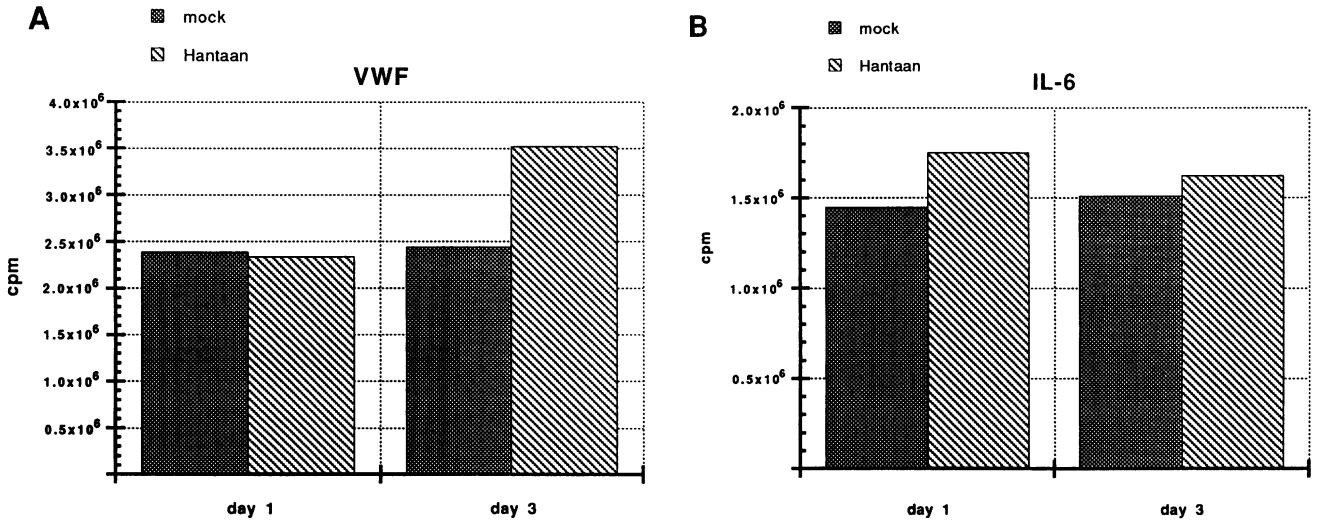

FIG. 6. Effect of HTV infection on the levels of IL-6 and vWF mRNA. RNA from mock-infected and HTV-infected HSVEC was harvested at either 1 day or 3 days postinfection. RNA was analyzed for the presence of mRNA by the PCR with specific primers. The final PCR products were assayed on a 2% agarose gel, transferred to Nytran, and probed with an internal ^{32}P-labelled oligonucleotide specific for either IL-6 or vWF. Samples were quantitated and then standardized against GAPDH mRNA. The experiments were run in duplicate, and the average values are shown; the standard deviation was not more than 5% for each point.

induction of interferon(s). Figure 7 shows that HTV infection of HSVEC results in substantially increased levels of IFN-β mRNA after 3 days of infection (23.5× the level found in mock-infected cells). No changes were seen in the levels of IFN-α mRNA as a result of HTV infection (data not shown).

Incubation of HTV-infected endothelial cells with anti-IFN antibody. Induction of a particular species of mRNA after infection with HTV does not necessarily mean that the protein is expressed in infected cells or that IFN is the cause of decreased virus yields in endothelial cells. To address these issues, HTV-infected endothelial cells were incubated in the presence of anti-IFN-β serum, and the yields of virus assayed by plaque assay were compared with those of untreated cultures. Table 1 shows that the presence of anti-IFN-β antibody dramatically affected the yield of HTV in endothelial cells between days 3 and 5. While control cultures (no anti-IFN antibody) gave decreasing yields of HTV (as observed earlier), the presence of the anti-IFN antibody allowed virus growth to reach the same range as in the permissive CV-7 cells. This same antiserum had no effect on HTV production in CV-7 cells, while control antiserum failed to stimulate production of HTV in infected endothelial cells (Table 1). We conclude that HTV infection of endothelial cells results in the induction of IFN-β, leading to the generation of an antiviral state restricting the production of HTV.

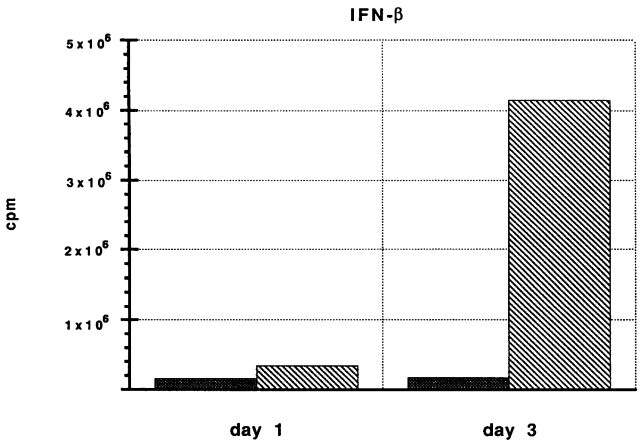

FIG. 7. Induction of IFN-β mRNA in HSVEC. RNA from mock-infected (▬) and HTV-infected (▨) HSVEC was harvested and processed as described in the legend to Fig. 6. RNA was analyzed for the presence of IFN-β mRNA by the PCR with specific primers. Samples were quantitated and standardized against GAPDH RNA. HTV-/mock-infected IFN-β mRNA ratios were 2.1 and 23.5 at days 1 and 3 postinfection, respectively. The experiments were run in duplicate, and the average values are shown; the standard deviation was not more than 5% for each time point.

TABLE 1. HTV growth in the presence of anti-IFN-β serum

Cell type[a]	Avg titer[b] on day postinfection	
	3	5
CV-1 with:		
No Ab	2.2×10^4	1.4×10^4
IFN-β Ab	2.7×10^4	1.2×10^4
Control Ab	3.0×10^4	1.2×10^4
HSVEC with:		
No Ab	1.0×10^3	2.0×10^2
IFN-β Ab	1.5×10^4	2.7×10^4
Control Ab	1.0×10^3	1.7×10^2

[a] Cell types used to grow HTV. Ab, antibody.

[b] Results represent the average titers of duplicate samples of medium removed either from HTV-infected cells which had been incubated with antiserum directly following adsorption (for day 3 results) or from HTV-infected cells on day 5 postinfection which had been reboosted on day 3 postinfection with an amount of antiserum equivalent to that given at day 0 (for day 5 results).

DISCUSSION

The results presented here strongly suggest that human endothelial cells isolated from both adult and fetal veins are highly susceptible to infection with HTV. However, in vitro infection with HTV does not lead to any noticeable cytopathic effect, as judged by both phase microscopy and electron microscopy. Thus, the pathogenesis resulting from HTV infection in humans may not be the result of direct viral damage to these cells but, perhaps, could be the consequence of some indirect mechanism, possibly immune mediated. Although human endothelial cells are clearly capable of supporting the growth of HTV, production of virus is limited to early times after infection and is followed by the establishment of an antiviral state, due to induction of IFN-β. Other proteins, such as GAPDH, vWF, and IL-6, are still produced, even in the absence of significant HTV production, suggesting that these cells are still viable.

This may imply that the human vascular system is not an active reservoir for infectious virus during the course of Korean hemorrhagic fever, and indeed, recent reports suggest macrophages as the target for virus spread in mice and humans, transporting virus efficiently to peripheral organs (1).

Our finding that IFN-β induction in endothelial cells inhibits production of infectious virus is consistent with the findings of Tamura et al. (29). Their work suggests that exogenously added human IFNs (IFN-α, -β, and -γ) all affect the replication of HTV in Vero E6 cells. Of the three, IFN-β inhibited virus replication most effectively.

The actual mechanism of interferon action on HTV growth in endothelial cells is not clear. A wide variety of studies has shown that type I IFNs inhibit virus replication via a number of mechanisms, all of which affect the ability of the virus to express its proteins effectively. One mechanism is the induction of a double-stranded RNA-dependent P1 protein kinase which catalyzes the phosphorylation of the alpha subunit of the protein synthesis initiation factor eIF-2 (25). Phosphorylation of eIF-2α has been shown to play an important role in the regulation of translation. A recent report showed that IFN treatment of a P1/eIF2α-deficient human fibroblast line was still able to prevent vesicular stomatitis virus replication, suggesting that human IFNs may be capable of inhibiting the replication of vesicular stomatitis virus in different types of human cells by different molecular mechanisms (34). In that context, we have found that 8-day-old HTV-infected endothelial cells which were not producing infectious virus still contained significant levels of viral glycoproteins and the nucleocapsid protein. Two plausible alternatives could explain this finding. First, HTV proteins may have an extremely long half-life, and/or second, IFN-β inhibition of HTV replication in endothelial cells occurs at some step in viral replication subsequent to translation of viral proteins.

An additional relevant question is the potential pathological consequence of IFN-β induction after human infection with HTV. For example, it has been suggested that IFN-α and IFN-β can cause destruction of virus-infected endothelial cells by increasing the expression of class I major histocompatibility complex antigens and making them targets for cytotoxic T lymphocytes (10, 23, 24). Such recruitment of cytotoxic T lymphocytes has been shown to involve activation of endothelial cell antigens, such as ELAM-1 and ICAM-1 (4). In addition, it is clear that the endothelium, which was once described as an inert blood barrier, now is known to play a dynamic role in a variety of proinflammatory and prothrombotic functions (19). This behavior is probably mediated through endothelial cells operating as both targets for and producers of cytokines, including IL-1 (7), Il-6 (19), and colony-stimulating factors (G-, GM-, and M-CSF) (24). It is interesting that IL-1, the most important cytokine for generation of fever, appears not to be induced as a consequence of HTV in our system. Additionally, neither IL-6 nor vWF appears to be induced by HTV infection. vWF has been shown to be a marker for endothelial cell damage and, as a consequence of its production, mediates platelet adhesion to damaged subendothelium.

The hallmark of HFRS has been described as a triad of fever, hemorrhage, and renal failure (6). It is not clear what effect HTV infection has on other specific endothelial cell genes involved in leukocyte recruitment, but our future work will be based on the hypothesis that these genes may play a role in the vascular injury seen in Korean hemorrhagic fever. Experiments in our laboratory are under way to examine which additional endothelial cell genes are regulated during HTV infection.

ACKNOWLEDGMENTS

We acknowledge Vernon Tesh (Microbiology Department, USUHS) for providing the human umbilical cells necessary for this work and Wendy Baur for performing harvest and primary culture of the human saphenous vein cells. We also acknowledge L. P. Perera for helpful discussions during the course of this work.

This work was supported by USPHS grant AI 25376 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Asada, H., M. Tamura, K. Kondo, Y. Dohi, and K. Yamanishi.** 1988. Cell-mediated immunity to virus causing haemorrhagic fever with renal syndrome: generation of cytotoxic T lymphocytes. J. Gen. Virol. **69**:2179–2188.
2. **Beaty, B. J., and C. H. Calisher.** 1991. Bunyaviridae—natural history. Curr. Top. Microbiol. Immunol. **169**:27–68.
3. **Bonthron, D., E. C. Orr, L. M. Mitscock, D. Ginsburg, R. I. Handin, and S. H. Orkin.** 1986. Nucleotide sequence of pre-provon Willebrand factor cDNA. Nucleic Acids Res. **14**:7125–7132.
4. **Cavender, D. E., D. Edelbaum, and L. Welkovich.** 1991. Effects of inflammatory cytokines and phorbol esters on the adhesion of U937 cells, a human monocyte-like cell line, to endothelial cell monolayers and extracellular matrix proteins. J. Leukocyte Biol. **49**:566–578.
5. **Chen, S.-Y., Y. Matsuoka, and R. W. Compans.** 1991. Assembly and polarized release of Punta Toro virus and effects of brefeldin A. J. Virol. **65**:1427–1439.
6. **Cosgriff, T.** 1991. Mechanisms of disease in hantavirus infection: pathophysiology of hemorrhagic fever with renal syndrome. Rev. Infect. Dis. **13**:97–107.
7. **Cozzolino, F., M. Torcia, D. Aldinucci, M. Ziche, F. Almerigogna, D. Bani, and D. M. Stern.** 1990. Interleukin 1 is an autocrine regulator of human endothelial cell growth. Proc. Natl. Acad. Sci. USA **87**:6487–6491.
8. **Dalrymple, J. M.** 1989. Plaque assay and plaque-reduction neutralization tests, p. 102–106. *In* H. W. Lee and J. M. Dalrymple (ed.), Manual of hemorrhagic fever with renal syndrome. WHO Collaborating Center for Virus Reference and Research Institute for Viral Diseases, Korea University, Seoul.
9. **Dveksler, G. S., A. Basile, and C. W. Dieffenbach.** 1992. Analysis of gene expression: use of oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase. PCR Methods Appl. **1**:283–285.
10. **Fajardo, L. F.** 1989. The complexity of endothelial cells. Am. J. Cell. Physiol. **92**:241–256.
11. **Gendelman, H., R. M. Friedman, S. Joe, L. M. Baca, J. A. Turpin, G. Dveksler, M. S. Meltzer, and C. W. Dieffenbach.** 1990. A selective defect of IFN production in HIV-infected monocytes. J. Exp. Med. **172**:1433–1442.

12. **Grcevska, L., M. Polenakovic, A. Oncevski, D. Zografski, and A. Gligic.** 1990. Different pathohistological presentations of acute renal involvement in Hantaan virus infection: report of two cases. Curr. Nephrol. **34:**197–201.
12a. **Hasty, S.** Unpublished observations.
13. **Jacobsen, H., J. Mestan, S. Mittnacht, and C. W. Dieffenbach.** 1989. Beta interferon subtype I induction by tumor necrosis factor. Mol. Cell. Biol. **9:**3037–3042.
14. **Kurata, T., T. F. Tsai, S. P. Bauer, and J. B. McCormick.** 1983. Immunofluorescence studies of disseminated Hantaan virus infection of suckling mice. Infect. Immun. **41:**391–398.
15. **Leduc, J. W.** 1989. Epidemiology of hemorrhagic fever viruses. Rev. Infect. Dis. **11:**S730–S734.
16. **Lee, P. W., C. J. Gibbs, C. D. Gajdusek, and R. Yanagihara.** 1985. Serotypic classification of Hantaviruses by indirect immunofluorescent antibody and plaque reduction neutralization tests. J. Clin. Microbiol. **22:**940–944.
17. **Lewis, R. M., H. W. Lee, A. F. See, D. B. Parris, J. S. Moon, D. J. Kim, and T. M. Cosgriff.** 1991. Changes in populations of immune effector cells during the course of haemorrhagic fever with renal syndrome. Trans. R. Soc. Trop. Med. Hyg. **85:**282–286.
18. **Mancuso, D. J., E. A. Tuley, L. A. Westfield, N. K. Worrall, B. B. Shelton-Inloes, J. M. Sorace, Y. G. Alevy, and J. E. Sadler.** 1989. Structure of the gene for human von Willebrand factor. J. Biol. Chem. **264:**19514–19527.
19. **Mantovani, A., and E. Dejana.** 1989. Cytokines as communication signals between leukocytes and endothelial cells. Immunol. Today **11:**370–375.
20. **Mantovani, A., A. Sica, F. Colotta, and E. Dejana.** 1990. The role of cytokines as communication signals between leukocytes and endothelial cells. Prog. Clin. Biol. Res. **349:**343–353.
21. **Pensiero, M. N., G. B. Jennings, C. S. Schmaljohn, and J. Hay.** 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. J. Virol. **62:**696–702.
22. **Ping, Z., and Y. Wei-song.** 1991. Effects of Hantaan virus on human endothelial cells and their significance in pathogenesis of hemorrhagic fever with renal syndrome. Chin. Med. J. **104:**924–929.
23. **Pober, J. S.** 1988. Cytokine-mediated activation of vascular endothelium. Am. J. Pathol. **133:**426–433.
24. **Pober, J. S., and R. S. Cotran.** 1990. Cytokines and endothelial biology. Physiol. Rev. **70:**427–451.
25. **Samuel, C.** 1991. Antiviral actions of interferon: interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology **183:**1–11.
26. **Schmaljohn, C. S., S. E. Hasty, S. A. Harrison, and J. M. Dalrymple.** 1983. Characterization of Hantaan virus, the prototype virus of hemorrhagic fever with renal syndrome. J. Infect. Dis. **148:**1005–1012.
27. **Schmaljohn, C. S., A. L. Schmaljohn, and J. M. Dalrymple.** 1987. Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order. Virology **157:**31–39.
28. **Sharefkin, J. B., K. D. Fairchild, R. A. Albus, D. F. Cruess, and N. M. Rich.** 1986. The cytotoxic effect of surgical glove powder particles on adult human vascular endothelial cell cultures: implications for clinical uses of tissue culture techniques. J. Surg. Res. **41:**463–472.
29. **Tamura, M., H. Asada, K. Kondo, M. Takahashi, and K. Yamanishi.** 1987. Effects of human and murine interferons against hemorrhagic fever with renal syndrome (HFRS) virus (Hantaan virus). Antiviral Res. **8:**171–178.
30. **Tannenbaum, S. H., and H. R. Gralnick.** 1990. γ-Interferon modulates von Willebrand factor release by cultured human endothelial cells. Blood **75:**2177–2184.
31. **Tesh, V. L., J. E. Samuel, L. P. Perera, J. B. Sharefkin, and A. D. O'Brien.** 1991. Evaluation of the role of shiga toxin and shiga-like toxins in mediating direct damage to human vascular endothelial cells. J. Infect. Dis. **164:**344–352.
32. **Xiao, S.-Y., R. Yanagihara, M. S. Godec, Z. A. Eldadah, B. K. Johnston, D. C. Gajdusek, and D. M. Asher.** 1991. Detection of Hantavirus RNA in tissues of experimentally infected mice using reverse transcriptase-directed polymerase chain reaction. J. Med. Virol. **33:**277–282.
33. **Yanagihara, R., and D. J. Silverman.** 1990. Experimental infection of human vascular endothelial cells by pathogenic and nonpathogenic hantaviruses. Arch. Virol. **111:**281–286.
34. **Zhang, X. T., and C. E. Samuel.** 1991. Mechanism of interferon action. Interferon alpha inhibits vesicular stomatitis virus primary transcript accumulation in P1/eIF-2α protein kinase-deficient human fibroblast cells. J. Biol. Regul. Homeostatic Agents **1:**157–165.