Persistent Seoul virus infection in Lewis rats

S. R. Compton, R. O. Jacoby, F. X. Paturzo, and **A. L. Smith**

Section of Comparative Medicine, Yale University School of Medicine, New Haven,
Connecticutt, U.S.A.

Summary. Mechanistic studies of hantavirus persistence in rodent reservoirs have been limited by the lack of a versatile animal model. This report describes findings from experimental infection of inbred Lewis rats with Seoul virus strain 80-39. Rats inoculated with virus intraperitoneally at 6 days of age became persistently infected without clinical signs. Tissues from Seoul virus-inoculated 6-day-old rats were assessed at 6, 9, and 12 weeks post-inoculation for viral RNA by RT-PCR and *in situ* hybridization (ISH) and for infectious virus by inoculation of Vero E6 cells. Virus was isolated from lung and kidney of infected rats at 6 weeks and viral RNA was detected in lung, kidney, pancreas, salivary gland, brain, spleen, liver and skin at 6, 9 and 12 weeks. Rats inoculated with Seoul virus intraperitoneally at 10 or 21 days of age became infected without clinical signs but had low to undetectable levels of viral RNA in tissues at 6 weeks post-inoculation. ISH identified vascular smooth muscle and endothelial cells as common sites of persistent infection. Cultured rat smooth muscle cells and to a lesser extent cultured endothelial cells also were susceptible to Seoul virus infection. Pancreatic infection resulted in insulitis with associated hyperglycemia. These studies demonstrate that infant Lewis rats are uniformly susceptible to asymptomatic persistent Seoul virus infection. Additionally, they offer opportunities for correlative *in vivo* and *in vitro* study of Seoul virus interactions in host cell types that support persistent infection.

Introduction

Hantaviruses are single-stranded RNA viruses in the family *Bunyaviridae*. Each of more than 20 known species has a natural rodent reservoir [28]. Human contact with infected rodents or their excreta [10] can lead to varied clinical outcomes ranging from asymptomatic to lethal infection [28, 38]. The global incidence of human hantavirus infection exceeds 150,000 cases per year, occurring primarily in Asia [32]. Additionally, more than 1,000 cases of hantavirus pulmonary syndrome,

a severe and often lethal condition, have been recorded in the United States, Canada, Panama and South America during the past 10 years [4].

The global distribution of hantaviruses reflects the broad range of their rodent hosts [32] with which they have co-evolved. Rural communities are at risk for strains transmitted by field rodents [5, 19, 31, 37], whereas both urban and rural communities are at risk for rat-borne hantavirus infection [17]. Urban risk also is favored in densely populated environments with inadequate rodent control [11] and refuse disposal. Laboratory acquired rat-borne hantavirus virus infections are rare with the last case of laboratory acquired Seoul virus infection reported in 1986 [23].

Persistence of hantaviruses in rodents increases risks for human exposure. Little is known about persistence mechanisms [25] and antiviral immunity appears insufficient to terminate infection expeditiously [33]. Study of persistent infection *in vivo* has been hampered by lack of a versatile animal model. This report demonstrates that Seoul virus (SEOV) infection of inbred Lewis rats may fill this need. It shows that Lewis rats are susceptible to persistent SEOV infection, that blood vessels are a major site of persistent infection, and that cultured vascular smooth muscle cells (SMC) and endothelial cells are susceptible to SEOV. These findings suggest new options for correlative *in vivo* and *in vitro* study of virus-host interactions, including influences of host immune factors. Additionally, we confirm that pancreatic islets are a site of persistent infection with resulting insulitis and hyperglycemia. Finally, we demonstrate that SEOV infects skin, which implies an additional source for natural exposure and transmission.

Materials and methods

Virus

SEOV strain 80-39 was obtained from Dr. Ho Wang Lee, Asian Institute for Life Sciences, Seoul Korea [20]. It had been passaged 3 times in Wistar rats and 6 times in Vero E6 cells prior to receipt. It was passaged two additional times in Vero E6 cells to produce a stock for animal inoculation.

Animals and animal inoculation

Pregnant inbred Lewis rats (*Rattus norvegicus*) were obtained from Harlan Sprague-Dawley, Inc (Indianapolis, IN). Serology performed prior to SEOV inoculation and at necropsy confirmed that rats were free of Sendai virus, pneumonia virus of mice, rat parvovirus, rat coronavirus and *Mycoplasma pulmonis*. They were housed in micro-isolator cages placed in HEPA-filtered Type IIB biological safety cabinets in quarters configured for animal studies with BSL 3 infectious agents. Rats were inoculated with SEOV on postpartum days 2, 6, 10 or 21. Suckling rats were inoculated intraperitoneally (i.p.) with 10^6 TCID50 of virus stock in 0.1 ml. Weanlings were anesthetized lightly with methoxyflurane and inoculated i.p. with the same dose. Control rats were inoculated with 0.1 ml of 80% Dulbecco's medium/10% Leibowitz 15 medium/10% fetal bovine serum. Rats were observed daily, euthanized at established time points or when clinical signs occurred. The Institutional Animal Care and Use Committee approved animal care and use.

Necropsy and tissue collection

Rats were necropsied at predetermined time points up to 12 weeks (84 days) after inoculation or at the onset of clinical signs. They were euthanized with methoxyflurane and cardiac blood was drawn for serology. Samples of lung, kidney, liver, salivary gland, pancreas and spleen were collected aseptically into sterile vials that were held in wet ice and then stored at $-80\,^{\circ}\mathrm{C}$ for virus isolation and assay by reverse transcriptase polymerase chain reaction (RT-PCR). Fragments of the same tissues, and brain, heart, intestine and skin were fixed in buffered formalin, embedded in paraffin wax and sectioned at $5\,\mu\mathrm{m}$ for histological staining and *in situ* hybridization.

Virus isolation

Once-thawed tissues were homogenized (10% tissue w/v) in 80% Dulbecco's medium containing 10% L15 media and 10% fetal bovine serum (Life Technologies, Rockville MD) and clarified by centrifugation within a laminar flow biological safety cabinet in a Picofuge$^{\mathrm{TM}}$ (Stratagene, La Jolla CA) for 1 min. Twenty-four-well plates containing confluent Vero E6 cells were inoculated with $200\,\mu\mathrm{l}$ of supernatant and held at $37\,^{\circ}\mathrm{C}$ for 2 h. Inocula were removed and cells were incubated in supplemented Dulbecco's medium (as above) in a humidified $37\,^{\circ}\mathrm{C}$, 5% CO_2 incubator for 12 to 13 days. Cultures were washed with phosphate-buffered saline (PBS) and trypsinized. Harvested cells were pelleted by centrifugation, re-suspended in PBS, about 2×10^4 cells were spotted onto Teflon-coated glass slides (Erie Scientific, Portsmouth, NH) and fixed briefly in cold acetone. SEOV antigen was detected by immunofluorescence staining with monoclonal antibody K-ECO2-BG01, which recognizes Hantaan virus nucleocapsid (N) protein, provided by Dr. Connie Schmaljohn (USAMRIID, Fort Detrick, MD) and fluorescein-conjugated goat anti-mouse IgG (Antibodies Inc, Davis, CA).

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from $50\,\mu\mathrm{l}$ of 10% homogenates of once-thawed tissues or Vero-E6 cell cultures using the RNeasy protocol (Qiagen, Chatsworth, CA). RT-PCR was performed on $5\,\mu\mathrm{l}$ samples of RNA using the Superscript One-Step RT-PCR System (Invitrogen, Carlsbad, CA). Primers specific for SEOV strain SR-11 M genome segment viral RNA (SR916:CCAGATTT CAGGGCAGATAGA and SR1738:CAATATGGGCACTCTCCTTGA) were synthesized by the W.M. Keck Foundation Biotechnology Laboratory, Yale University. The reaction cycle was: 30 min at $50\,^{\circ}\mathrm{C}$; 2 min at $94\,^{\circ}\mathrm{C}$; 40 cycles of 30 sec at $94\,^{\circ}\mathrm{C}$, 60 sec at $48\,^{\circ}\mathrm{C}$, 120 sec at $68\,^{\circ}\mathrm{C}$; 10 min at $72\,^{\circ}\mathrm{C}$. RT-PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide and the 842 base pair amplicon was visualized by ultraviolet illumination.

In situ hybridization (ISH)

The S segment of SEOV strain SR-11 cloned into the Bluescript KS plasmid (Stratagene, La Jolla, CA) was provided by Dr. Connie Schmaljohn (USAMRIID, Fort Detrick, MD). Randomly primed DNA probes were prepared from the S segment, labeled with ^{32}P or ^{35}S following the manufacturer's protocol (New England Biolabs, Beverly, MA), and purified in low melting point agarose. Tissue sections were prepared, hybridized and developed as previously described [2]. A cell was scored as positive if the overlying grain count was at least 8 grains (twice background). Microscopic evaluation of radiolabeled sections was based on a semi-quantitative scale. Signal prevalence was defined as greater than 100 positive cells per tissue section (+++), 50–100 cells positive per tissue section (++), 5–50 positive

cells per tissue section (+), 1–5 positive cells per tissue section (+/−) or negative (−). Biotinylated S segment probes also were made using linearized plasmid and protocols supplied by the manufacturer (NEN Life Science Products, Boston, MA) and labeled with horseradish peroxidase (HRP). Sections were hybridized and developed, using tyramide amplification and the reaction product was assessed by light microscopy [12]. Positive (known SEOV-infected) and negative (known virus-free) control tissues were included with each ISH procedure.

Immunostaining

Tissue sections were stained for pancreatic β cells using a polyclonal guinea pig antibody against swine insulin (Dako Corporation, Carpinteria, CA) and HRP-conjugated goat anti-guinea pig antibody (Vector Laboratories, Burlingame, CA) and visualized using 4,4 di-aminobenzidine as substrate.

Serology

SEOV antibodies were detected by ELISA using 75 ng/well of bacterially expressed re-combinant histidine-tagged SEOV N protein. Antibodies were detected using goat anti-rat Ig, anti-rat IgG or anti-rat IgM linked to HRP, and 3,3′,5,5′-tetramethylbenzidine as substrate (Kirkegaard and Perry, Gaithersburg, MD). Negative controls were wells containing 75 ng/well of bacterially expressed recombinant histidine-tagged β-galactosidase or PBS and wells incubated with normal rat serum. Positive control wells were stained with known SEOV-positive rat serum.

Rat smooth muscle cell (SMC) cultures

Primary SMC cultures isolated from rat aorta were obtained from Dr. Stephen Pang of Queens University, Kingston, Ontario [29]. Cells were seeded into 4-well glass chamber slides (Nalge Nunc International, Naperville, IL) and maintained in Dulbecco's medium with low glucose, 10% calf serum, 8 mM HEPES buffer, 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies, Rockville MD). Initial cultures reached confluence after incubation at 37 °C for 5 days. Cells were fusiform to polygonal and positive for α actin by immunostaining. They were inoculated with SEOV 80-39 at a multiplicity of infection of 0.5 as described above and observed daily for cytopathic effect (CPE). Cultures were fixed in acetone at established time points and immunostained for SEOV antigen using convalescent sera from Seoul virus infected rats and fluorescein-conjugated goat anti-rat IgG. Monolayer cultures of Vero E6 cells were inoculated with stock virus as positive controls and uninoculated SMC and Vero E6 cells served as negative controls. Additionally, virus-inoculated SMC were subjected to immunofluorescence staining using normal rat serum as the primary antibody.

Rat endothelial cell (EC) cultures

EC cultures were obtained from Dr. Joseph Madri of Yale University. Cells were seeded into 4-well glass chamber slides (Nalge Nunc International) and maintained in 60% Dulbecco's medium, 30% L15 medium 10% fetal bovine serum with 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies). Cultures were inoculated with SEOV 80-39 at a multiplicity of infection of 0.5 as described above and observed daily for cytopathic effect (CPE). Cultures were fixed in acetone at established time points and immunostained for SEOV antigen using convalescent sera from Seoul virus strain 80-39 infected rats and fluorescein-conjugated goat anti-rat IgG. Monolayer cultures of Vero E6 cells were inoculated with stock virus as positive controls and uninoculated EC and Vero E6 cells served as negative controls. Additionally, virus-inoculated EC were subjected to immunofluorescence staining using normal rat serum as the primary antibody.

Measurement of glucose concentrations

Fasting serum glucose concentrations (mg/dl) were determined on heat inactivated (56 °C for 30 min) serum from SEOV infected and uninfected rats by Antech Diagnostics (Farmingdale, NY).

Results

Induction of persistent infection

In situ hybridization (ISH) detected systemic infection in all age groups by day 7 (Table 1). Infection remained asymptomatic except in pups inoculated at 2 days of age, which developed severe neurological signs about a month after inoculation due to disseminated infection of the brain (data not shown). Table 1 also shows that the prevalence of SEOV-infected tissues was higher in rats inoculated at day 6 compared to day 10 or 21 and that infection persisted for at least 6 weeks. Therefore, an additional group of rats was inoculated at 6 days of age and examined through 12 weeks, during which they sustained infection. Additional findings for these two groups are described collectively and in greater detail below.

Detection of persistent SEOV infection

SEOV-positive cells were detected by ISH in lungs and kidneys of all rats examined at 6 and 9 weeks (Table 2). Therefore, these tissues were tested for infectious virus and by RT-PCR for viral RNA. Lungs and kidneys from all rats at 6 weeks contained infectious virus (Table 2). No infectious virus was detected in 10 rats tested at 9 weeks, despite the fact that their lungs and kidneys were uniformly SEOV-positive by RT-PCR. Thus, molecular hybridization was more sensitive than *in vitro* culture for detecting SEOV under the conditions employed. SEOV

Table 1. Effect of age on induction of persistent Seoul virus infection as determined by in situ hybridization[a]

Age at inoculation (days)	Prevalence of virus-positive tissues[b,c] at week after inoculation			Clinical signs
	1	3	6	
2	++ (11)	++ (4)	++ (4)	+
6	++ (8)	++ (4)	++ (4)	−
10	+ (10)	nd	+/− (5)	−
21	+/− (10)	nd	− (4)	−

[a]^{32}P-labeled, randomly-primed SEOV probe

[b]Mean prevalence of virus-positive cells in submandibular/sublingual salivary gland, lung and kidney as defined by semi-quantitative criteria: +++ (greater than 100 positive cells per tissue section), (++) (50–100 positive cells per tissue section), (+) (5–50 positive cells per tissue section), (+/−) (1–5 positive cells per tissue section) or (−) negative. nd = not determined

[c]Numbers in parentheses indicate number of rats examined

Table 2. Detection of Seoul virus by virus isolation, RT-PCR or in situ hybridization (ISH) in tissues of Lewis rats inoculated at 6 days of age

Method	Tissue	No. of virus-positive tissues at week after inoculation[a]		
		6	9	12
Virus isolation	lung	4/4	0/4	nd[b]
	kidney	4/4	0/4	nd
RT-PCR	lung	nd	5/6	5/6
	kidey	nd	6/6	6/6
	pancreas	nd	2/6	3/6
	salivary gland	nd	nd	1/4
ISH	lung	4/4	6/6	3/6
	kidney	4/4	6/6	6/6
	pancreas	4/4	6/6	6/6
	salivary gland	nd	nd	4/4

[a]Number of positive tissues/ number of tissues tested
[b]Not determined

RNA was not detected in control rats inoculated with tissue culture media (data not shown).

Forty samples of lung, kidney, pancreas and salivary gland collected from 6 rats per time point at 9 and 12 weeks were then tested for SEOV RNA (Table 2). It was detected in all rats, but a higher proportion of lungs and kidneys were SEOV RNA-positive (22/24), compared to pancreas and salivary gland (6/16). There was strong concordance between RT-PCR and ISH results, but some samples positive by RT-PCR were negative by ISH and vice-versa. However, 39 of 40 tissues collected at 9 and 12 weeks were SEOV-positive by one or both methods.

Distribution and lesions of SEOV infection

Infection was detected by ISH through 12 weeks (Table 3). The prevalence of RNA-positive cells per tissue varied per timepoint and decreased gradually after 3 weeks post-inoculation (Table 4). Signal was prominent in muscular blood vessels of multiple tissues during acute and persistent infection. ISH with tyramine-amplification confirmed that SMC and endothelial cells were affected in both phases of infection (Fig. 1a). Virus-positive SMC also were found in muscle tunics of intestine and urinary bladder, but mucosal infection was not observed there or elsewhere. By contrast, parenchymal cells in many tissues contained viral RNA during acute and persistent infection. These included sites conducive to virus excretion: liver, lung (Fig. 1b), salivary gland (Fig. 1c), exocrine pancreas and kidney. Viral RNA was also found in skin, including vessels of the subcutis, the *panniculus carnosus* muscle (Fig. 1d) and epidermis (Fig. 1e). Although neurological signs were not observed, viral RNA-positive neurons and glial cells were found in cerebrum and cerebellum (data not shown).

Table 3. Prevalence of Seoul virus-positive tissues in rats inoculated at 6 days of age as determined by in situ hybridization[a]

Tissue	Weeks after inoculation				
	1	3	6	9	12
Brain	nd[b]	nd	4/4	9/10	6/6
Pancreas	nd	5/8	4/4	10/10	6/6
Lung	4/4	7/9	4/4	10/10	3/6
Kidney	4/4	7/8	4/4	9/10	6/6
Salivary gland	4/4	7/9	4/4	10/10	6/6
Spleen	3/4	1/1	4/4	8/10	2/6
Liver	3/3	6/9	2/4	6/10	2/6
Skin	nd	2/5	4/4	9/10	2/2

[a]Number of positive tissues / number of tissues tested
[b]Not determined

Table 4. Prevalence of Seoul virus-positive cells in tissues of rats inoculated at 6 days of age as determined by in situ hybridization[a]

Tissue	Weeks after inoculation				
	1	3	6	9	12
Brain	nd[b]	nd	++	++	++
Lung	+++	+++	++	++	+
Kidney	++	++	++	++	+
Pancreas	nd	++	++	++	++
Salivary gland	+	+	+	+	+
Spleen	+	+	+	+	+/−
Liver	+/−	+/−	+/−	+/−	+/−
Skin	nd	+/−	+	+/−	+/−

[a]See legend for Table 1
[b]Not determined

Necrosis was minimal, even in heavily infected tissues. However, mononuclear cell infiltrates were present by the third week especially adjacent to renal vessels bearing virus-positive SMC and in pancreatic islets (Fig. 1f). Focal interstitial inflammation occurred sporadically in lung. No lesions were found in the kidney, pancreas or lung of control rats inoculated with tissue culture medium (data not shown).

Insulitis and hyperglycemia

Pancreatic islets also were infected by day 7 followed within 2 weeks by insulitis characterized by influx of mononuclear cells that resulted in partial effacement of islets (Fig. 1g). However, all affected islets contained at least some

β cells. Additionally, all rats with insulitis had SEOV-positive islet cells. No inflammation was detected in exocrine pancreas despite virus-positive acinar cells.
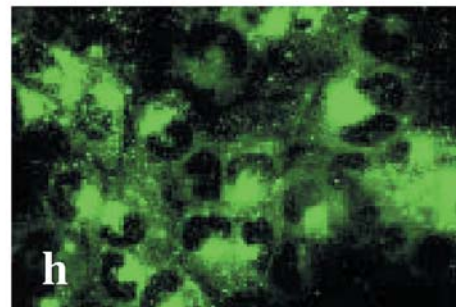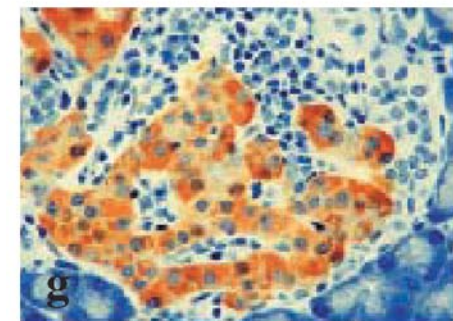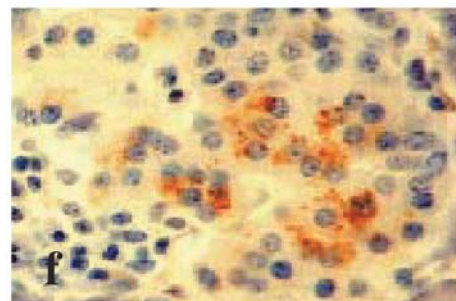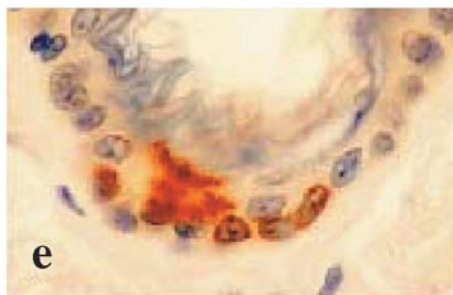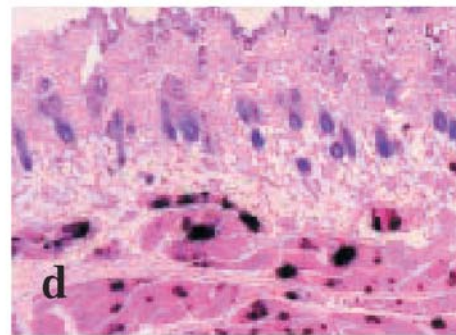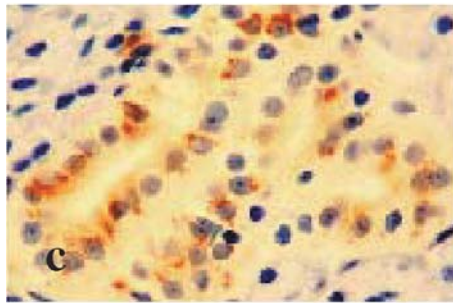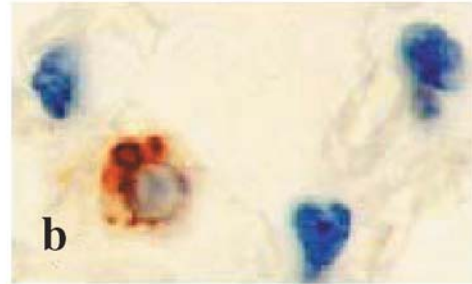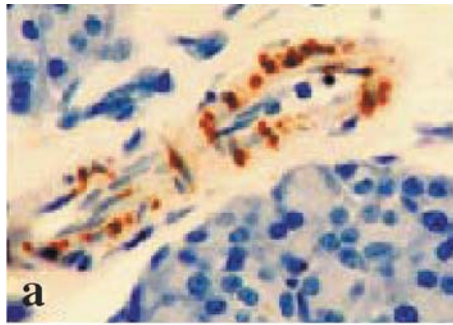
Table 5. Correlation of Seoul virus infection with insulitis and hyperglycemia

PI week	Rat no.	SEOV RNA in islets	Insulitis	Fasting serum glucose (mg/dl)
9	1	+	+	101
	2	+	+	257
	3	+	+	297
	4	+	+	99
	5	+	−	89
12	1	−	−	160
	2	+	+	152
	3	−	−	160
	4	+	+	112
	5	−	−	168
	6	+	+	148
12	4 controls	−	−	82–121

To determine the potential effects of insulitis on serum glucose a third group of rats was inoculated with SEOV at six days of age and tested 9 and 12 weeks later. Table 5 shows that 40% of rats at 9 weeks and 84% of rats at 12 weeks had developed hyperglycemia. Correlation between glucose values and insulitis was positive, but incomplete. Thus, two rats tested at 9 weeks had insulitis with normal blood glucose levels, whereas 3 rats tested at 12 weeks had elevated blood glucose without insulitis.

Serology

All rats tested at 3 weeks and thereafter had serum antibodies to SEOV (26/26). IgG antibodies were detected in all seropositive rats and IgM was detected in

Fig. 1. In situ hybridization (**a–f**) demonstrating Seoul virus (SEOV) RNA in tissues of persistently infected Lewis rats inoculated with SEOV 80-39 at 6 days of age. Panels **a,b,c,e,f**, were hybridized by tyramide amplification using a biotinylated probe to the S segment of SEOV giving a brown reaction product. Panel **d** was hybridized using a ^{32}P-labeled S segment probe. Panel **g** was immunohistochemically stained for pancreatic β cells using a polyclonal guinea pig antibody against swine insulin and HRP-conjugated goat anti-guinea pig antibody. Panel **h** demonstrates immunofluoresence staining using convalescent sera from Seoul virus infected rats and fluorescein-conjugated goat anti-rat IgG. **a**: Submandibular salivary gland 9 weeks after inoculation. SEOV in SMC and endothelium of an arteriole. **b**: Lung 12 weeks after inoculation. SEOV in an alveolar pneumocyte. **c**: Submandibular salivary gland 12 weeks after inoculation. SEOV in epithelium of salivary ducts. **d**: Skin 9 weeks after inoculation. SEOV in the *panniculus carnosus* muscle layer. **e**: Skin 9 weeks after inoculation. SEOV in epidermis. **f**: SEOV in Pancreatic islet cells. Mononuclear cells are infiltrating at the lower left. **g**: Immunostain of a pancreatic islet for β cells 12 weeks after inoculation with SEOV. There also is a prominent mononuclear cell infiltrate and the β cell population is partially effaced. **h**: Monolayer culture of rat smooth muscle cells infected with SEOV and immunostained to illustrate intracytoplasmic SEOV antigen

all but one rat. Five uninoculated control rats held through week 12 remained seronegative.

Susceptibility of SMC and EC cultures to SEOV

Because SEOV infection was prominently vasculotropic *in vivo*, we investigated whether SMC and EC were susceptible to virus in culture in an effort to develop *in vitro* correlates to study SEOV host cell interactions. SEOV antigen was detected in about 5 percent of cultured SMC by day 3 after inoculation and in more than 50 percent of cells by day 9 (Fig. 1h). Although some infected cells swelled, no cytolytic changes were detected through this time point. Both infected and uninfected SMC cultures began to deteriorate by day 12 and were discarded by day 15. In contrast to SMC, EC had low susceptibility to infection. SEOV antigen was detected in scattered cells 2 and 4 days after inoculation. Cultures of both infected and uninfected EC had deteriorated by day 6.

Discussion

Our ISH results show that infant Lewis rats inoculated with SEOV 80-39 develop asymptomatic, persistent infection in multiple tissues including sites conducive to virus excretion: lung, salivary glands, and kidney. This aspect of our study confirms previous results of experimental hantavirus infection in rats [14, 36]. Although we did not observe intestinal infection, infection of the liver and pancreas could lead to fecal infectivity by excretion of bile from infected liver and enzymes from infected pancreas. The new finding of SEOV viral RNA in skin, by contrast, should provoke reassessment of risks for transmission of infection. ISH demonstrated that skin infection was persistent and involved epidermis, dermal and subcuticular vasculature, and myofibers of the *panniculus carnosus*, the underlying muscle layer. Additional studies are needed to determine if SEOV RNA in skin represents infectious virus. If so, it would broaden potential routes for viral transmission by aggressive behavior such as biting first proposed by Glass and co-workers [9]. While bite wounds could produce viremia directly, they also could result in local viral replication in skin. Conversely, skin infection could contribute to virus transmission through oral contamination of aggressor animals. While epidermal infection also may enhance risks for contact transmission or environmental contamination through exfoliation of keratinocytes, such cells are usually non-viable and therefore unlikely to harbor infectious virus.

Differences in tissue distribution, virus excretion and duration of infection after experimental inoculation among various reports of experimental hantavirus infection highlight that various host and viral factors influence the expression of infection [16, 18, 21, 22]. For example, viral antigen was widely disseminated in newborn and 6 week-old Wistar rats inoculated with several strains of SEOV (SR-11, KI-262 or TB-314) or Hantaan virus 76-118 [15]. Inoculation of adult rats caused persistent infection in some animals, with viral antigen detected, at

varying prevalence, in a more restricted distribution (lung, spleen, liver and heart) [15]. Lee et al. reported that virus shedding in urine and feces of infected rats occurred with Hantaan virus strain 76-118 but not with SEOV 80/39 [22]. SEOV has been reported to persist in young rats for 6 months whereas it persists in adult rats less than 2 months [13]. The duration of infection, not surprisingly, also is affected by immune status. Thus, immunodeficient nude rats are more susceptible to SEOV infection than immunocompetent rats [7]. Lastly, gender also may affect susceptibility since male rats were reported be more likely to shed virus in saliva or by multiple routes than were female rats [16]. These variables also suggest that more than one model will be required to investigate mechanisms of persistent infection comprehensively.

Hantaviruses are known to be endotheliotropic in rats [34, 36] but we demonstrated that SMC are prominent sites of acute and persistent infection. Vascular infection exhibited a shift from endothelium to SMC during the transition from acute to persistent infection. A similar shift occurs in persistent parvovirus infection of rats, which also is vasculotropic [12], and suggests that vascular infection begins in endothelium, as a result of viremia, then extends to subjacent SMC. Alternatively, viral injury to endothelium could lead to direct exposure of SMC by leakage of virus-infected plasma through compromised intercellular junctions. Our results also demonstrated that SMC and, to a lesser extent, endothelial cells are susceptible to SEOV infection *in vitro*. Therefore, factors influencing the onset and duration of SEOV infection should be amenable to correlative study *in vivo* and *in vitro*.

Insulitis, accompanied by hyperglycemia was a common feature of persistent SEOV 80-39 infection. A similar finding was reported by Lee and co-workers for SEOV infection of F344 rats [21] but was not illustrated with data or photomicrographs. Similarly, Yamanouchi and coworkers found that newborn rats inoculated with SEOV strain B-1 developed antigen-positive pancreatic islet and acinar cells, but blood glucose levels were not reported [36]. Our immunostaining results suggest that insulitis was associated with β cell loss, but this observation will require quantification. The correlation between insulitis and hyperglycemia was not absolute, which suggests that both could be transient. However, they were detected as late as 12 weeks post-inoculation. Prolonged inflammation could increase risks of functional damage to endocrine pancreas. It remains to be seen whether SEOV-induced chronic insulitis leads to a diabetes-like condition with attendant increase in water consumption and urination that could affect the amount or duration of virus excretion.

Although infection of 6-day-old rats was asymptomatic, pathogenic neurological infection occurred in infants inoculated at 2 days of age. Neurovirulence is common after experimental inoculation of hantavirus strains into very young rats or mice, especially with brain-adapted stocks [35, 36] and cautions against intracerebral passage for the preparation of inocula. Nevertheless, our finding illustrates that even stocks without a prominent history of brain passage have the capacity for neurovirulence.

Molecular hybridization was more sensitive for detecting SEOV than was virus isolation from frozen and thawed tissues, a result reported during studies

of other RNA viruses [30]. There are several potential causes for this variance. Low virus concentrations typical during persistent infections may go undetected after small losses in infectivity during cryopreservation or subsequent dilution of samples during preparation of inocula. Cryopreservation and thawing also can reduce infectivity by rupturing viral envelopes with loss of receptors for binding to target cells. Lastly, it has been suggested that virus can be neutralized by exposure to antibodies during preparation of tissues for tissue culture inoculation [28]. For these reasons, immediate cultivation of test suspensions with indicator cells may be preferable for detecting infectious SEOV in persistently infected tissues. We found that detection of virus is favored by molecular amplification methods, such as RT-PCR. However, these methods detect both infectious and non-infectious viral particles.

The mechanisms of persistent hantavirus infection are obscure. Meyer and Schmaljohn [25] recently reviewed potential factors favoring persistent infection which included: immature host immunity at the onset of infection, immune dysfunction caused by hantavirus infection of antigen presenting cells, antigenic variation of infecting virus to avoid immunological detection, and down-regulation of virus replication in host cells. For example, it has been suggested that SEOV infection of macrophages and lymphocytes could prolong infection by reducing immune responses to virus [26]. SEOV replication generates terminally deleted viral RNAs that have been postulated to downregulate viral gene expression thereby facilitating persistence [24]. This may help to explain why pre-emptive administration of antibody can protect rats from SEOV infection, whereas administration of antibody after the initiation of infection is ineffective [6, 40].

Adoptive cell transfer studies performed in mice imply a protective role for cell-mediated immunity against hantavirus infection. Transfer of unsorted or B cell-depleted immune splenocytes protected neonatal BALB/c against challenge with Hantaan virus [27]. Additionally, transfer of immune sera or T cells from BALB/c mice into athymic mice protected them from challenge with Hantaan virus [1] and transferred immune splenocytes protected adult SCID mice against Hantaan virus infection [39]. Comparable experiments have not been reported for the rat. However, development of mononuclear cell infiltrates at some sites of infection (e.g. kidney, pancreas) in our studies is consistent with activation of cell-mediated immunity. Its functional effectiveness must be assessed, including why inflammatory infiltrates do not develop in many persistently infected tissues. The gradual postpartum maturation of immunity in rats, which can take up to several months, may bear on this issue. It suggests that persistent infection is favored in rats infected before achieving full immune competence. The use of Lewis rats should facilitate definition of host immunity in SEOV persistence since they have been extensively characterized immunologically [8]. Moreover, they provide the potential to examine persistent infection and host responses to virus correlatively *in vitro* and *in vivo*, using the cell type (SMC) which serves as the major target for persistent infection. Other models of hantavirus persistence in their natural rodent reservoir appear less versatile than the Lewis rat model of SEOV infection. For example, the recently described deer mouse (*Peromyscus*)

model of Sin Nombre virus persistence utilizes an uncommon laboratory rodent for which immunological and cell culture based reagents are scarce [3].

Acknowledgements

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Author's address: Susan R. Compton, Section of Comparative Medicine, Yale University School of Medicine, PO Box 208016, New Haven, CT 065 20-8016, U.S.A., e-mail: susan.compton@yale.edu