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Extreme Susceptibility of African Naked Mole Rats (*Heterocephalus glaber*) to Experimental Infection with Herpes Simplex Virus Type 1

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Herpes simplex virus type 1 (HSV1) is widely used as a gene delivery vector in a variety of laboratory animals. In a recent study, a thymidine-kinase–inactive (replication-conditional) HSV1 used as a delivery vector was lethal in naked mole rats, whereas mice infected with the identical virus showed no adverse effects. This result prompted us to undertake a controlled comparative histologic study of the effect of HSV1 infection on naked mole rats and mice. Replication-competent and replication-conditional HSV1 caused widespread inflammation and necrosis in multiple organ systems of naked mole rats but not mice; naked mole rats infected with replication-defective virus showed no adverse effects. We conclude that the lethality of HSV1 for naked mole rats is likely the result of overwhelming infection, possibly in part due to this species' natural lack of proinflammatory neuropeptides at the initial site of infection.

Abbreviation: HSV1, herpes simplex virus type 1.

Herpes simplex virus type 1 (HSV1) belongs to the *Simplexvirus* genus of the *Alphaherpesvirineae* subfamily and is an important human pathogen.[21] Similar to other herpesviruses, HSV1 is well adapted to its natural host. Fatal HSV1 infections of immunocompetent humans are relatively rare. In most cases, human HSV1 infections lead to lifelong latent infection that is interrupted by episodes of viral reactivation.[32] Experimental infection of mice, rabbits, rats, and guinea pigs has been used widely to study HSV1 pathogenesis.[33] The pathogenesis of HSV1 in these animals shows close resemblance to infections seen in humans. Infection of peripheral tissues leads to local viral replication and brief viremia. The virus also spreads by neural pathways to the peripheral and central nervous systems, where virus again may replicate, this time in neurons and nonneuronal cells, and may cause encephalitis. Animals surviving the acute phase of infection do not demonstrate signs of encephalitis, and infectious virus is no longer detectable in their nervous system or other organs. However, HSV1 usually is not cleared from these animals and typically establishes latency in neurons of sensory ganglia.

Various HSV1 isolates possess a number of characteristics that make them promising as vectors for gene delivery.[7] These properties include their capacity to package large amounts of heterologous DNA and an ability to establish persistent, lifelong infections, during which the viral genome remains as a circular nonintegrated episome. In addition, HSV1-based vectors can infect a wide range of human cell lines and primary cultures with high efficiencies. This attribute allows HSV1-based vectors to stably transduce neurons and provide sustained heterologous gene expression. As such, HSV1-based vectors offer the characteristics of an artificial chromosome combined with a highly efficient delivery system. HSV1 strains used for gene therapy typically are engineered to have decreased virulence; for example, strains with defective viral thymidine kinase cannot replicate in nervous tissue, will not cause encephalitis, and are avirulent to immunocompetent hosts.[8]

Naked mole rats have been used to study pain because they do not produce substance P and calcitonin gene-related peptide from the C fibers in their skin[17] and they lack C-fiber–related responses to capsaicin.[18] In other mammals, these peptides play important roles in pain signaling in the spinal cord and in initiating local immune responses in the periphery.[15,19] We infected naked mole rats with a thymidine-kinase–inactivated (replication-conditional) HSV1 engineered to express the preprotachykinin gene that encodes the pain-related neuropeptides substance P and neurokinin A.[4] Viruses used in the comparative study did not carry transgenes.

Case Report

Male and female 8-mo-old naked mole rats from an inhouse breeding colony (n = 10 total) received 1.0×10^8 PFU replication-conditional HSV1 in 25 µL applied to a scarified area of the dorsal tarsus. The rodents recovered from the procedure uneventfully until day 10 after the procedure, when 1 animal was found dead and 2 were lethargic and ataxic; these latter 2 rodents were euthanized and necropsied. Tissues evaluated by histology and immunocytochemistry included adrenal gland, bladder, bone marrow, brain, cecum, colon, duodenum, heart, ileum, inoculated foot, jejunum, kidney, liver, lung, pancreas, sciatic nerve, salivary gland, sex organs, skin, spinal cord at multiple levels, spleen, stomach,

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thymus, thyroid gland, and trachea. The remaining 7 inoculated animals died less than 24 h after the first was found dead.

On gross examination, the livers were pale, and the spleens were small. Microscopically, widespread herpetiform intranuclear inclusions were present in the liver, with diffuse lymphoid necrosis involving the lymph nodes, spleen and thymus. Immunocytochemistry specific for HSV1 proteins[31] revealed widespread expression in the liver, spleen, colon, small intestine, lymphatic tissue, and lung. Less extensive, focal HSV1 protein expression was found in the heart, kidney, salivary gland, and brain. The antigen was present in all necrotic tissues.

Approximately 50% to 80% of hepatocyte nuclei contained herpetiform inclusions. Most nuclei contained 'ground-glass' inclusion bodies, with distention of the nuclear membrane. Some nuclei contained acidophilic Cowdry type A intranuclear inclusion bodies, which are composed of intact and disrupted virions with peripheral margination of the nuclear chromatin. Widespread single-cell necrosis was present but no focal areas of parenchymal necrosis. The sinusoidal spaces contained increased numbers of leukocytes, and endothelial cell prominence was increased slightly. The portal areas were normal. However, focal perivenous cytoplasmic vacuolation of the hepatocytes surrounded some of the central veins. Overall, hepatocytes had normal cell density and uniform volume across the functional lobule.

The spleens had widespread necrosis of the periarteriolar lymphoid sheaths with complete destruction of lymphoid follicles. The areas of necrosis consisted of apoptotic cellular debris with an influx of vesicular mononuclear cells. The sinus areas were congested and contained focal areas of extramedullary hematopoiesis, primarily erythroid.

The lymph nodes had an absence of germinal centers with multiple aggregates of tingible-body macrophages. One cervical thymic remnant had medullary atropy and widespread cortical necrosis with numerous large tingible-body macrophages.

The microscopic lesions in the liver spleen, lymph nodes, and thymus were consistent with severe, systemic HSV1 infection.

Materials and Methods

Design of the comparative study. We designed a controlled comparative study to assess the susceptibility of naked mole rats to HSV1. Groups of 2 female 7-mo-old Hsd:ICR mice (Harlan, Madison, WI) and a 10-mo-old nonbreeding male or female naked mole rat were inoculated with replication-competent, replication-conditional, or replication-defective HSV1.

Animals. This study was approved by the Animal Care Committee at the University of Illinois at Chicago, an AAALAC-accredited institution. The naked mole rats were born in the facility from breeders acquired from Dr J Jarvis (University of Capetown, South Africa); they were housed as described.[1] The mice were housed in autoclaved static microisolation caging, provided sterile water, and fed irradiated chow (LM485 Mouse–Rat Diet 7912, Teklad, Madison, WI). Mice and mole rats were housed in facilities free of mouse parvovirus, mouse hepatitis virus, murine norovirus, Theiler murine encephalomyelitis virus, mouse rotavirus, Sendai virus, pneumonia virus of mice, reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, *Ectromelia virus*, K virus, mouse polyoma virus, mouse thymic virus, mouse cytomegalovirus, Hantavirus, *Encephalitozoan cuniculi*, cilia-associated respiratory bacillus, ectoparasites and helminth endoparasites.

Viruses. The low-virulence KOS strain[27] was the parental HSV1 used to create the recombinant viruses in this study. Virus was produced in Vero cells (CCL81, American Type Culture Collection, Manassas, VA). The replication-defective, ICP4$^-$ mutant of HSV1 used was d120.[6] Production of replication-defective recombinant viruses was performed by using the ICP4-complementing 7B cell line generously provided by JC Glorioso (University of Pittsburgh, PA).[14] For generation of replication-competent and replication-defective virus, an *Ase*I–*Afl*II fragment of pIRES2–EGFP (Clontech, Mountain View, CA) was blunt-cloned into the *Hin*dIII site of a shuttle plasmid containing HSV1 nucleotides 80176 to 81324 (Figure 1). The transferred fragment contained an expression cassette including the human cytomegalovirus immediate-early enhancer–promoter, encephalomyocarditis virus internal ribosome entry site, enhanced green fluorescent protein gene, and simian virus 40 polyadenylation site (PA). The *Escherichia coli lacZ* gene, obtained as a *Not*I fragment from pCMVβ (Clontech) was blunt-cloned into the *Sma*I site of the shuttle contruct. Homologous recombination after linearization of the shuttle plasmid and digestion of KOS (replication-competent) or d120 (replication-defective) DNA with *Spe*I occurred after cotransfection of the DNAs into Vero or 7B cells, respectively.[13] The viruses were purified to homogeneity by limiting dilution.[9] Stocks of purified viruses were produced in the appropriate cell line, concentrated by high-speed centrifugation, and stored at –80° in 10% sucrose in isotonic physiologic balanced saline (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4).

For generation of the replication-conditional HSV1, an expression cassette consisting of the human cytomegalovirus immediate-early enhancer–promoter, a *Pac*I linker, and the simian virus 40 polyadenylation site was cloned into the *Sna*BI site of a shuttle plasmid containing HSV1 nucleotides 46752 to 48634 and the *E. coli lacZ* gene with flanking *Pac*I sites; the shuttle construct subsequently was cloned into the *Pac*I site of the expression cassette (Figure 1). Insertion of the expression cassette into the thymidine kinase gene prevents expression of the enzyme and renders the virus incapable of replication in nondividing cells, such as neurons.[25,26]

Viral inoculation and euthanasia. Mice were anesthetized with 80 mg/kg ketamine and 4 mg/kg xylazine intraperitoneally; naked mole rats were anesthetized with 50 mg/kg ketamine and 3 mg/kg xylazine intraperitoneally. The dorsal tarsus of the right foot was scarified, and 10.0×10^8 PFU of replication-competent, replication-conditional, or replication-defective HSV1 in 25 µL was applied topically. All animals were anesthetized on day 7 after inoculation, exsanguinated, and necropsied. Tissues sampled included brain, cecum, colon, duodenum, heart, ileum, inoculated foot, jejunum, kidney, liver, lung, pancreas, spinal cord at multiple levels, spleen, and stomach. Histologic sections of tissues were evaluated in a blind manner.

Histologic methods. Tissue sections either were stained with hematoxylin and eosin or were examined by immunohistochemistry using a polyclonal HSV1-specific antiserum as previously described.[31] Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 3 to 5 µm. Tissue sections examined by immunohistochemistry were deparaffinized with xylene and rehydrated through a series of graded ethanols. Endogenous peroxidase activity was quenched by using a 0.3% H_2O_2–methanol bath followed by several washes with isotonic phosphate-buffered saline (pH 7.4). HSV1 antigens were detected

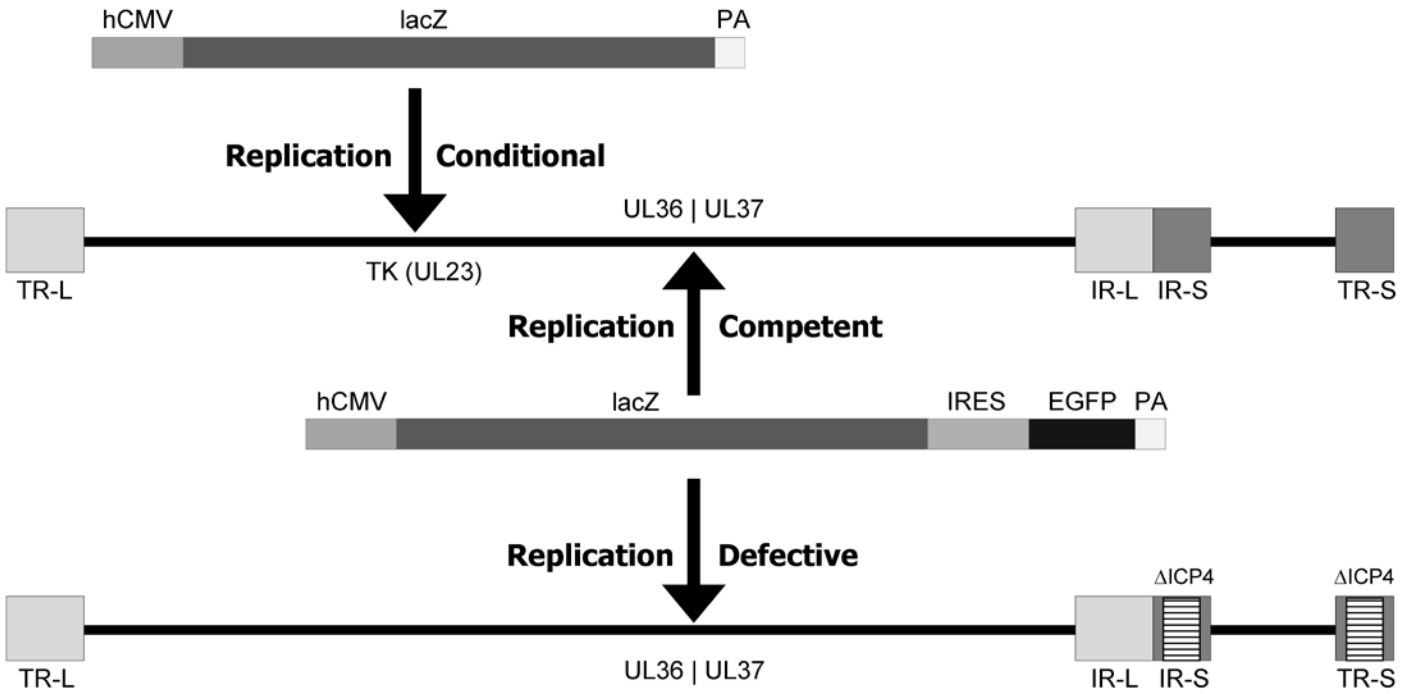

Figure 1. The creation of replication-conditional, replication-competent, and replication-defective viruses. For replication-conditional virus, an expression cassette was inserted into the HSV1 thymidine kinase [TK(UL23)] gene of HSV1 strain KOS. Insertion of the expression cassette into the thymidine kinase gene prevents expression of the enzyme and renders the virus incapable of replication in nondividing cells, such as neurons. For replication-competent virus, an expression cassette was inserted HSV1 strain KOS between the UL36 and UL37 genes. This virus was capable of replication in all tissues but was considered to have low virulence. For replication-defective virus, an expression cassette was inserted into the $ICP4^-$ mutant of HSV1 between the UL36 and UL37 genes. This virus was incapable of replication.

by using a 1:1000 dilution of a rabbit HSV1-specific antiserum (Dako, Carpinteria, CA). Incubation with primary antibody at 43 °C for 32 min was followed by the addition of biotinylated antirabbit immunoglobulin secondary antibody, avidin–horseradish peroxidase, and 3,3′-diaminobenzidine tetrahydrochloride (0.04%) in 0.05 M Tris-HCl (pH 7.4) and 0.025% H_2O_2 as a chromogen (Ventana Medical Systems, Tucson, AZ). Before staining, binding of secondary antibodies and conjugates was blocked by appropriate reagents provided by the manufacturer.

Results

The naked mole rats inoculated with replication-competent HSV1 had visually small spleens, mild multifocal chronic dermatitis at the inoculation site, widespread viral inclusions in the liver (Figure 2 A) associated with HSV1 immunoreactivity (Figure 2 B), moderate to severe acute multifocal splenitis with generalized disruption of lymphoid follicles (Figure 2 C) associated with HSV1 immunoreactivity (Figure 2 D), moderate diffuse acute mesenteric lymphadenitis, moderate focal acute enteritis with viral inclusions, and mild multifocal interstitial pneumonia.

Approximately 80% to 90% of the hepatocytes contained herpetiform inclusions. Most nuclei contained ground-glass intranuclear inclusion bodies, with a smaller number of nuclei containing acidophilic Cowdry type A intranuclear inclusion bodies. Severe diffuse cytoplasmic vacuolation of the hepatocytes was present, with small aggregates of inflammatory cells, primarily neutrophils, scattered throughout the hepatic parenchyma.

The splenic sinuses were infiltrated with large numbers of neutrophils and macrophages. The white pulp showed widespread loss of lymphoid follicles, with intrafollicular necrosis and necrosis of the lymphoid sheaths surrounding the central arterioles. The areas of necrosis consisted of apoptotic cellular debris, vesicular mononuclear cells, and tingible-body macrophages. The red pulp contained focal areas of suppurative necrosis and aggregates of extramedullary hematopoiesis. The interstitial regions of the lung were multifocally infiltrated with moderate numbers of macrophages and neutrophils and fewer lymphocytes and plasma cells, resulting in thickened alveolar walls.

The small intestines of naked mole rats inoculated with replication-competent HSV1 had focal areas of severe degeneration and necrosis involving the crypt epithelial cells and mesenchymal cells within the lamina propria. Small numbers of these cells contained herpetiform intranuclear inclusions of both the Cowdry type A and ground- glass types (Figure 2 E). The gut-associated lymphoid tissue showed moderate necrosis, characterized by apoptotic cellular debris, vesicular and tingible-body macrophages, and few to moderate numbers of neutrophils (Figure 2 F). The mesenteric lymph nodes had lesions similar to those of the gut-associated lymphoid tissue.

Macroscopically, naked mole rats inoculated with replication-conditional HSV1 had visually large spleens. Microscopically, the animals that received the replication-conditional HSV1 had moderate to severe ulcerative and necrosuppurative dermatitis at the inoculation site, mild focal acute nonsuppurative hepatitis (Figure 3 A) associated with HSV1 immunoreactivity (Figure 3 B), a generalized decrease in the size and number of splenic follicles

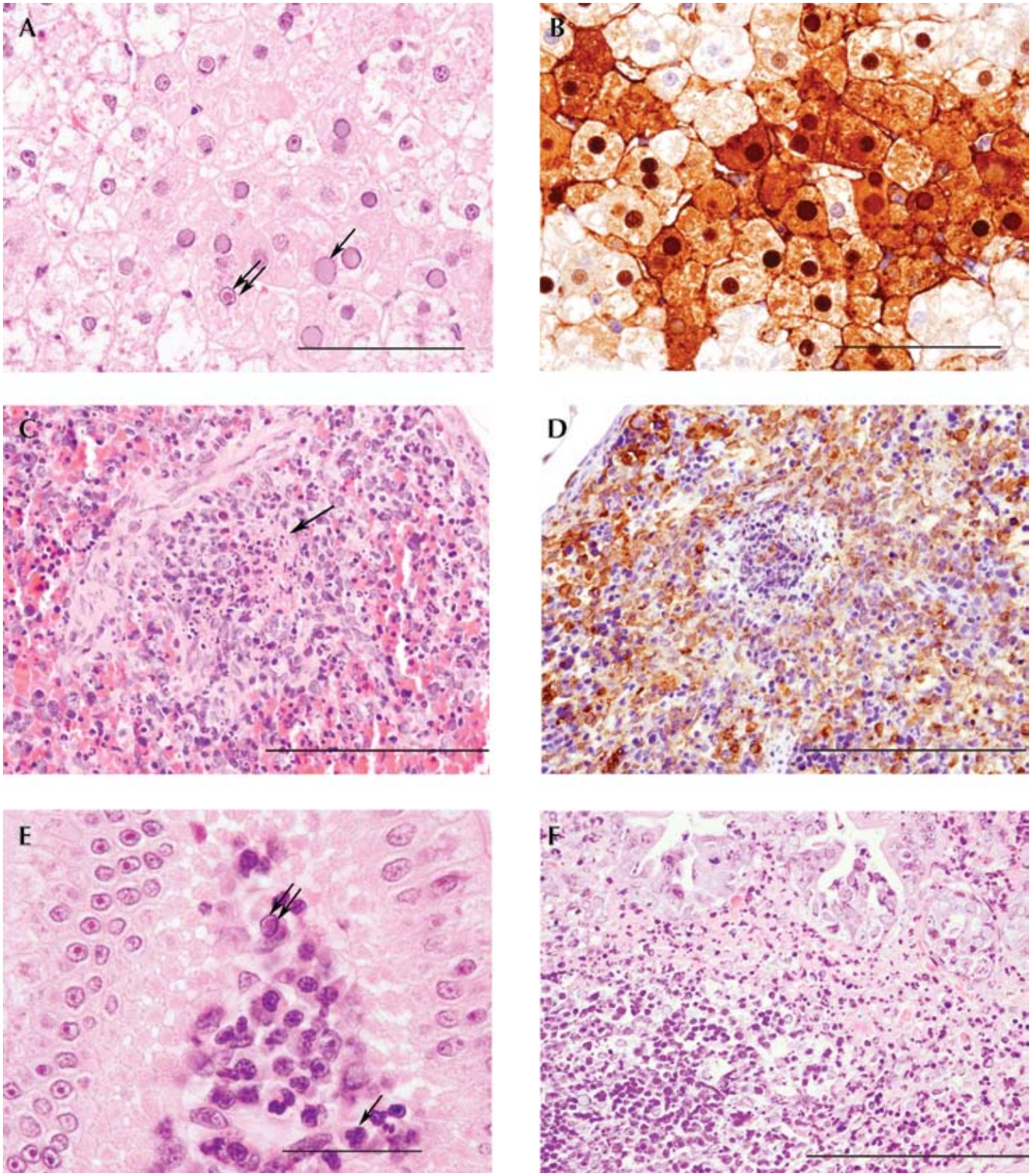

Figure 2. Photomicrographs of a naked mole rat that received replication-competent HSV1. (A) Large numbers of ground-glass (black arrow) and small numbers of Cowdry type A (double black arrow) intranuclear inclusion bodies in the liver. Hematoxylin and eosin stain; bar, 100 µm. (B) Large numbers of immunoperoxidase-positive hepatocytes. Note the intracytoplasmic and intranuclear staining of the cells. Avidin–horseradish peroxidase stain with hematoxylin counterstain; bar, 100 µm. (C) Acute splenitis with severe follicular degeneration and necrosis (black arrow). Hematoxylin and eosin stain; bar, 200 µm. (D) Note the diffuse immunoperoxidase-positive staining of lymphocytes and mononuclear cells. Avidin–horseradish peroxidase with he-

associated with HSV1 immunoreactivity (Figure 3 C, D), and mild focal acute enteritis with necrosis of the gut-associated lymphoid tissue (Figure 3 E).

Specifically, the liver of naked mole rats inoculated with replication-conditional HSV1 had diffuse cytoplasmic vacuolation of hepatocytes, with focal areas (3 or 4 cells) of necrosis. Focal small aggregates of inflammatory cells consisting of variable numbers of neutrophils were scattered throughout the parenchyma. The spleen had widespread loss of lymphoid follicles and a marked decrease in the number of lymphocytes surrounding the central arterioles. The dilated red pulp sinuses contained large numbers of red blood cells, neutrophils, macrophages, and foci of extramedullary hemotopoiesis. The small intestine had diffuse necrosis of the duodenal gut associated lymphoid tissue characterized by apoptotic cellular debris, vesicular and tingible body macrophages, and moderate numbers of neutrophils.

Macroscopically and microscopically, the naked mole rat that was inoculated with replication-defective HSV1 had no noteworthy lesions or HSV1 immunoreactivity.

None of the inoculated mice showed any gross lesions at necropsy. The mice inoculated with replication-competent HSV1 had severe focally extensive erosive and ulcerative necrosuppurative dermatitis at the inoculation site and mild focal lymphocytic meningitis of lumbar spinal cord associated with HSV1 immunoreactivity. The mice inoculated with replication-conditional and replication-defective HSV1 showed no noteworthy histopathologic changes or immunoreactivity.

These findings indicate that compared with mice, naked mole rats are far more susceptible to disseminated HSV1 infection.

Discussion

Naked mole rats are used increasingly in a wide variety of laboratory studies because of their unusual ecology, anatomy, and physiology.[2,3,5,11,17,18,20,22] One aspect of the recent study involved infection of the skin on the foot with replication-conditional HSV1 engineered to express the preprotachykinin gene that encodes the pain-related neuropeptides substance P and neurokinin A.[4] All 10 of the naked mole rats inoculated died. Subsequently, we confirmed the susceptibility of naked mole rats to replication-conditional HSV1 without transgenes for substance P and neurokinin A.

Naked mole rats were very susceptible to infection with replication-competent and replication-conditional HSV1—all of mole rats with either of these viruses developed systemic infections, with the most notable histologic lesions in the liver, spleen, and lymphatic tissue. In addition, viral antigens were found in the colon, small intestine, lymphatic tissue, spleen, lung, heart, kidney, and salivary gland. The brain demonstrated only minimal involvement.

We chose the day 7 time point for ending the study because we hypothesized that replication-competent HSV1 would be more virulent than the replication-conditional strain, and we surmised that the replication-conditional HSV1 was responsible for killing the naked mole rats at 10 d after infection. None of the naked mole rats were clinically ill at 7 d after infection, but those that received replication-competent HSV1 had more widespread infection, as evidenced by immunohistochemical findings.

Compared with mice, naked mole rats were dramatically more susceptible to both replication-competent and -conditional HSV1s. Numerous published reports indicate that inoculation of HSV1 into peripheral organs like skin or cornea of adult mice typically leads to a self-limited, nonlethal infection.[21,31,33] As expected, even mice inoculated with replication-competent HSV1 strain in our current study did not show evidence of severe systemic infection. However, histopathologic evidence of infection was detected at the site of virus inoculation and in the nervous system. Mice inoculated with either replication-conditional or -defective HSV1 showed no evidence of nervous system pathology or of systemic viral disease. However, the degree of inflammation at the initial site of replication in the foot varied between mice and naked mole rats, in that the inflammation was most severe in the mice that received the replication-competent HSV1. However, naked mole rats that received replication-competent HSV1 did not have marked inflammation due to a poor response at the local site of infection[24] (data not shown). Irrespective of this possibility, our study clearly demonstrates a striking susceptibility of naked mole rats to HSV1.

We can only speculate on the reason for the high susceptibility of naked mole rats to the detrimental effects of HSV1-induced infection. In the wild, these animals are isolated from other mammalian species due to their fully subterranean lifestyle, so they may not have evolved protective mechanisms to other mammalian pathogens. For example, Asian macaques are very susceptible to simian hemorrhagic fever virus, a virus found in African monkeys.[16] Naked mole rats developed lethal coronavirus infection that was attributed to inbreeding.[23] Our colony of naked mole rats likely is highly inbred because we have no management schemes to increase their heterozygosity. Compared with other rodents, naked mole rats live extremely long lives (20 y and more).[3] They have a low metabolic rate and high thermal conductance,[2] both of which may influence their longevity. Yet the kinetics of the viral infection in terms of time until death was similar to those of other immunocompetent rodents given lethal viral infections.

HSV1 causes fatal infections in severe combined immunodeficient (SCID) mice.[28-30] However, in contrast to the fairly rapid lethal affects in naked mole rats, death for SCID mice occurs several weeks to months after peripheral (corneal) inoculation of a thymidine-kinase-deficient strain of HSV1.[30] This faster lethal time course in NMR is also more typical of fatal HSV1 infections in people.[10] People that develop fatal disseminated HSV1 infections are typically patients with hematologic and lymphoreticular malignancies, have AIDS or congenital immunodeficiencies, or are organ or bone graft recipients. Children are also susceptible. Disseminated infections in humans affect the liver, spleen, and lung, in descending order of frequency. As mentioned earlier, naked mole rats are a unique mammal in that they lack secretion of substance P and calcitonin gene-related peptide from nerve fibers in the skin. Perhaps this or some other mechanism allows the virus to replicate to higher levels at the initial site of infection. Additional studies would help elucidate the pathogenic mechanisms.

matoxylin counter stain; bar, 200 µm. (E) Infiltration of the villous lamina propria with medium numbers of neutrophils (black arrow). Note the Cowdry type A intranuclear inclusion body in a mononuclear cell (double black arrow). Hematoxylin and eosin stain; bar, 50 µm. (F) Moderate necrosis of gut-associated lymphoid tissue and mononuclear cells within the villous lamina propria of the small intestine. Hematoxylin and eosin stain; bar, 200 µm.

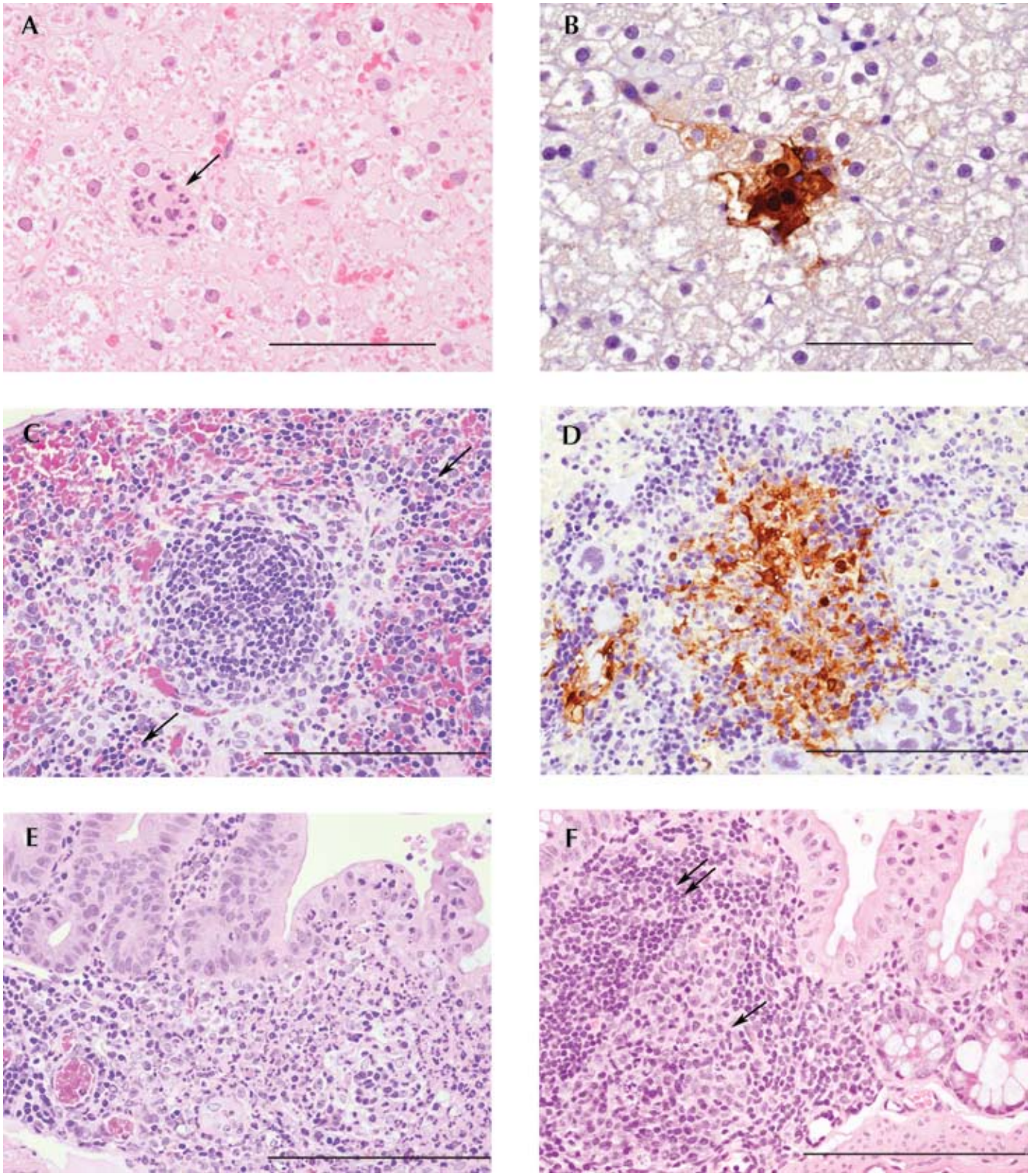

Figure 3. Photomicrographs of a NMR that received replication-conditional HSV1. (A) Focal neutrophilic infiltration (black arrow) with mild diffuse vacuolation and increased granularity of the hepatocytes. Note the absence of intranuclear inclusion bodies. Hematoxylin and eosin stain; bar, 100 µm. (B) Focal immunoperoxidase-positive staining hepatocytes. Avidin–horseradish peroxidase stain with hematoxylin counterstain; bar, 100 µm. (C) Small splenic follicle with no distinct germinal center and decreased lymphocytes in the mantel and marginal zones. Note the extramedullary hematopoiesis

The pathologic changes observed in the naked mole rats were indicative of an overwhelming viral infection. Most lymphatic tissues showed marked necrosis associated with HSV1 antigen. However, the liver showed little inflammation despite evidence of viral infection in virtually every cell. The cause of death likely was a systemic inflammatory response syndrome caused by overwhelming infection.[12]

In summary, we have documented unique susceptibility of naked mole rats to HSV1 infection. Naked mole rats develop systemic, fatal infection after inoculation with strains of HSV1 that are replication-conditional (that is, have an inactive thymidine kinase) and are avirulent for immunocompetent mice. The virulence of replication-competent HSV1 was greater than that of replication-conditional HSV1. Our clinical and histopathologic findings suggest that naked mole rats are extremely susceptible to HSV1 and that HSV1 will cause overwhelming systemic infection in these animals. Care should be taken when working with herpesviruses in and around naked mole rats.

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in the red pulp surrounding the follicle (black arrows). Hematoxylin and eosin stain; bar, 200 µm. (D) Note the immunoperoxidase staining of lymphocytes and mononuclear cells within the splenic follicle. Avidin–horseradish peroxidase with hematoxylin counter stain; bar, 200 µm. (E) Focal acute enteritis with mild to moderate suppurative necrosis of gut-associated lymphoid tissue in the small intestine. Hematoxylin and eosin stain; bar, 200 µm. (F) Photomicrograph of the small intestine of a naked mole rat that received replication-defective HSV1. Note the normal gut-associated lymphoid tissue in the small intestine with a germinal center (black arrow) and thick mantle of small lymphocytes (double black arrow). Hematoxylin and eosin stain; bar, 200 µm.

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