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The Cotton Rat Provides a Novel Model To Study Genital Herpes Infection and To Evaluate Preventive Strategies

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Prevention of genital herpes and other sexually transmitted infections (STI) is a critical health priority because of the overwhelming impact on women and infants and the epidemiological association with human immunodeficiency virus (HIV)/AIDS. Small animal models are essential for evaluating strategies for prevention or treatment of STI. Neither the murine nor the guinea pig model of genital herpes fully recapitulates human disease. We demonstrate that herpes simplex virus type 2 (HSV-2) readily infects inbred cotton rats (*Sigmodon hispidus*). Consistent infection does not require pretreatment with medroxyprogesterone, and primary disease resembles that observed in humans. The animals develop genital lesions and fully recover. During primary infection, viral DNA is also detected in liver, lungs, brain, and kidneys. Clinical self-limited recurrences occur spontaneously but may also be induced by dexamethasone. Pretreatment of cotton rats with PRO 2000 gel, a candidate vaginal microbicide being evaluated in clinical trials to prevent HSV and HIV, protects cotton rats from HSV. Together, these studies suggest that the cotton rat may provide an excellent model to study genital herpes and to evaluate preventive strategies.

Genital herpes is one of the most prevalent sexually transmitted infections (STI) worldwide and is associated with substantial morbidity. Population-based studies in developing countries show herpes simplex virus type 2 (HSV-2) seroprevalence rates ranging from 60 to 80% in young adults (15). What makes HSV infections so difficult to control is that most sexual and vertical transmission occurs during unrecognized or asymptomatic shedding (15). Epidemiological studies consistently demonstrate that genital herpes enhances the transmission of human immunodeficiency virus (HIV) (7). HSV may facilitate HIV acquisition by disrupting epithelial cells, thereby increasing exposure to HIV target cells, and may modulate the mucosal environment by activating proinflammatory or suppressing protective factors (14, 26). The increasing prevalence of genital herpes, the frequent recurrence of clinical episodes, the high frequency of asymptomatic shedding, and the association of HSV with HIV transmission highlight the urgent need for control measures in populations at risk for both viruses.

One preventive strategy is the development of topical microbicides, self-administered agents designed for vaginal use to prevent STI. Goals for the development of topical microbicides are that they be safe; affordable; effective against HIV, HSV, and other STI; and stable in the presence of genital tract secretions. A key component to the preclinical development of topical microbicides is evaluation of safety and efficacy in small animal models that reflect human disease. Most small animal studies of the pathogenesis of and preventive therapies for genital herpes have been conducted using murine models. Advantages include small size, inexpensiveness, the availability of large numbers of inbred strains and gene knockout strains, and a wide range of reagents available for study. However, the mouse is not an optimal model of human disease. First, consistent infection requires treatment with medroxyprogesterone to induce a diestrous phase (25). Hormonal treatment may be important for thinning of the vaginal mucosa but also may influence innate and adaptive immune responses to the virus (10, 11). Human infection and clinical or subclinical reactivations appear to occur independently of the menstrual cycle (4). Second, genital herpes infection of mice spreads to the paracervical ganglia, which are the major autonomic ganglia of the pelvis, resulting in urinary and rectal distention, hind-limb paralysis, and death, outcomes not observed in human disease (24). The high rates of mortality preclude studies of horizontal and vertical transmission and of the impact of preventive strategies on the establishment of latency or frequency of clinical and subclinical reactivations.

An alternative model that permits studies of recurrent disease is the guinea pig (31, 32). The guinea pig model more closely resembles human disease but also has substantial limitations. Disadvantages include the paucity of inbred strains and limited availability of immunologic reagents. This is particularly relevant to studies designed to test the safety of candidate topical microbicides because a model that enables investigators to carefully evaluate the effects of repeated applications of microbicides on the mucosal environment and innate immunity is critical.

The cotton rat, a common New World rodent, in a family distinct from the common laboratory mouse and rat, is a well-established model of several human viral diseases, most notably, respiratory syncytial virus, measles, and parinfluenza virus type 3 (21–23, 27–29, 36–39). For reasons that remain unclear,

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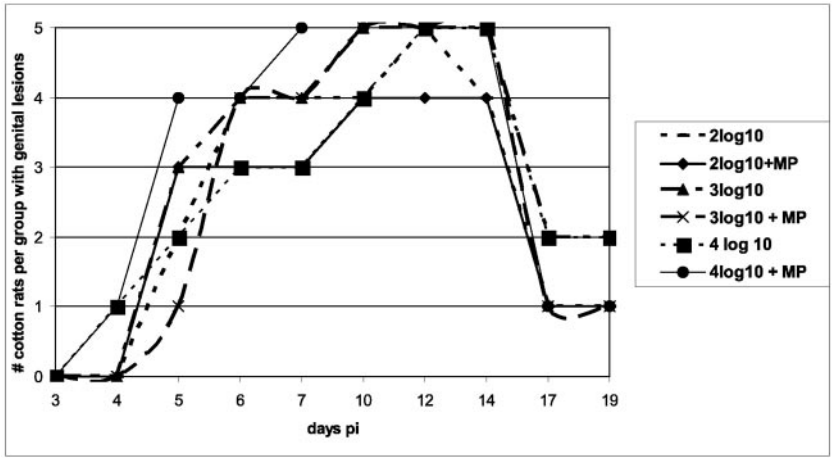

FIG. 1. HSV induces genital tract lesions as early as 4 days p.i., independent of inoculum dose or treatment with medroxyprogesterone (MP). Results are the number of cotton rats in each group (five rats per group) with clinically evident genital tract lesions at the indicated time p.i.

the cotton rat is susceptible to human viruses where murine models have failed. The importance of this animal as a model of several clinically important human diseases has led to the recent development of reagents that will permit study of mucosal immunity in this animal model (1, 2). Recently, we found that the cotton rat is an excellent model of HSV-1 infections of the lip (herpes labialis) (16). Building on these observations, we explored the potential role of cotton rats as a model of genital herpes.

MATERIALS AND METHODS

Intravaginal inoculation of cotton rats. Inbred female *Sigmodon hispidus* cotton rats (Virion Systems, Inc., Rockville, MD) were maintained and handled under veterinary supervision in accordance with National Institutes of Health guidelines and the Virion Institutional Animal Care Utilization Committee. For inoculations, 6-week-old female cotton rats (~80 g each) were anesthetized with ketamine (10 mg/kg of body weight) and xylazine (6 mg/kg). The vaginal vault was gently swabbed with a wet calcium alginate-tipped swab and then a dry calcium alginate-tipped swab to remove mucus, and then groups of 20 rats were inoculated with a sterile cotton swab containing 10^2, 10^3, or 10^4 PFU of HSV-2(G) inserted into the vagina for 30 min. Six cotton rats were mock infected and served as controls. The animals were observed daily for 3 weeks and twice weekly for an additional 10 weeks. Alternatively, cotton rats were inoculated with 30 μl of virus suspension (10^3, 10^4, or 10^5 PFU per animal) using a pipette. In the first experiment only, cotton rats were pretreated with a single intramuscular dose of 4-mg/kg medroxyprogesterone 24 h prior to infection.

Recovery of infectious virus and detection of virus by PCR. Vaginal swabs were obtained with a premoistened cotton swab on days 1, 2, 3, 4, 5, 7, 10, 14, and 21 postinfection (p.i.). The applicators were placed directly into sterile microtubes containing 0.5 ml minimal essential medium with antibiotics and Fungizone (Cambrex, Walkersville, MD) and stored at −80°C. HSV titers were determined by standard plaque assay of Vero cells grown in 96-well plates. In addition, two animals per group were sacrificed on days 1, 3, 5, 7, 10, 14, 21, and 100, and the following tissues were isolated and flash frozen for PCR: lumbosacral cord and dorsal root ganglia, brain, liver, lung, and kidney. Control animals were sacrificed on day 100. DNA was isolated from dissected tissues using a DNA isolation kit (DNeasy Tissue kit; QIAGEN, Valencia, CA), according to the manufacturer's specifications. The HSV thymidine kinase (TK) gene was amplified by nested PCR using *Taq* DNA polymerase (Platinum *Taq* DNA polymerase; Invitrogen, Carlsbad, CA). In the first PCR round, DNA samples

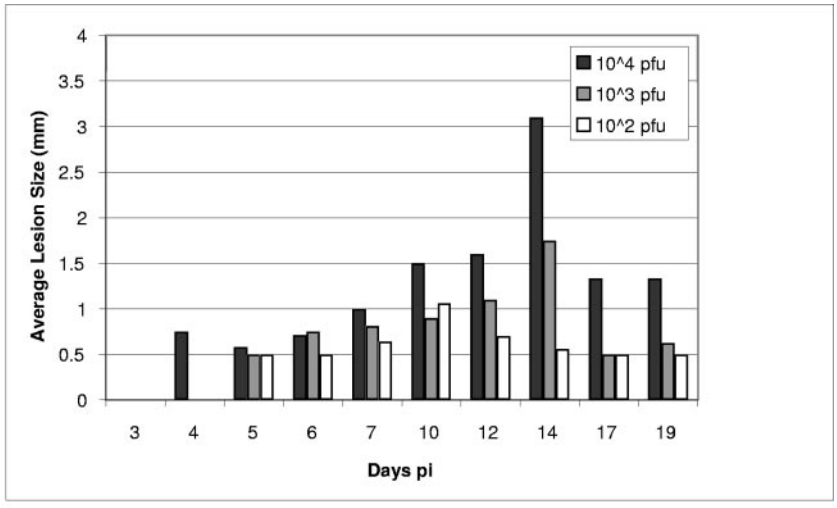

FIG. 2. The average size of the lesions (in millimeters) per infected cotton rat during primary HSV infection peaks on day 14 p.i. and increases with inoculum dose. Results are geometric means of the sizes of the lesions from six infected animals per group per time.

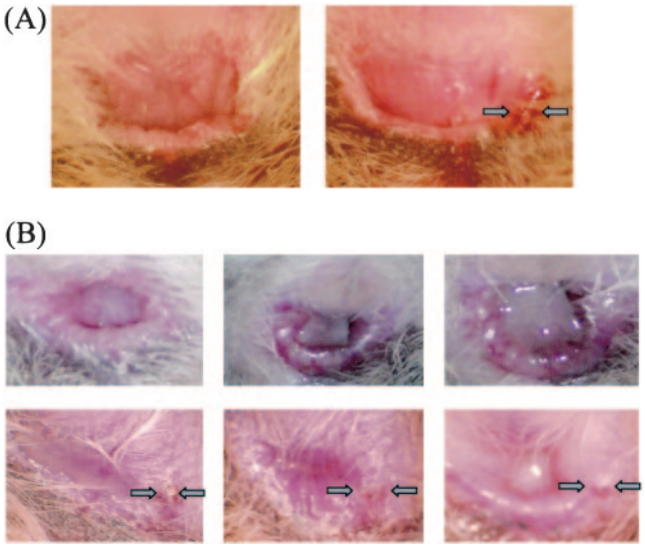

FIG. 3. Representative photographs illustrating primary and spontaneous or dexamethasone-induced recurrent genital tract lesions. (A) Cotton rat inoculated with 10^5 PFU HSV-2, shown 1 day postinfection (left) and the same cotton rat 35 days p.i.; arrows indicate a spontaneous recurrent ulcer. (B) Representative photographs of cotton rats during primary and dexamethasone-induced recurrences. Top left, mock infected, 7 days p.i.; top middle, 10^4 PFU of HSV-2, 7 days p.i; top right, 10^4 PFU of HSV-2, 14 days p.i.; bottom left, recurrence 12 days post-dexamethasone treatment; bottom middle, recurrence 18 days post-dexamethasone treatment; bottom right, recurrence 21 days post-dexamethasone treatment.

(200-ng aliquots) were incubated with HSV TK-specific primers (5′-AATCGC GAACATCTACACCAC-3′ and 5′-AAAGCTGTCCCCTTACCTCCC-3′) and subjected to 30 PCR cycles (each cycle consisting of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min, except in the last cycle, which was 72°C for 12 min). For the second round of PCR, 3 μl of the first-round reactions was incubated with the primers 5′ CTGCAGATACCGCTCCGTATT-3′ and 5′-CATCTTCGACCG CCATCCCAT-3′ and subjected to 30 PCR cycles under the same conditions as the first cycle. For reaction controls, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified using nested PCR as previously described (16). The amplified products were analyzed by agarose gel electrophoresis. The HSV TK-amplified gene product was 266 bp, and GAPDH was 230 bp.

PRO 2000 protection study. Female cotton rats were pretreated with 4% PRO 2000 gel, a matched placebo gel, or phosphate-buffered saline (PBS) (15 animals per group). Indevus Pharmceuticals, Lexington, MA, provided the gels (18, 34). Animals were anesthetized, and a moistened cotton swab was used to clean the mucosal lining. A 1.0-ml syringe was used to load the gel and a 0.15-ml volume was inserted into the vaginal vault. Fifteen minutes later, 10 rats in each group were intravaginally inoculated with 0.1 ml HSV-2(G) at 10^5 PFU/ml; the other 5 rats were mock infected with PBS as controls. The animals were swabbed daily for 30 days and twice a week for additional 30 days. On days 3, 7, and 14, two animals from each infected groups were sacrificed and brain, liver, lung, kidney, and vaginal tissues were removed for PCR and histology.

RESULTS

Cotton rats are susceptible to genital HSV infection independent of progesterone therapy. To determine if female cotton rats are susceptible to intravaginal infection and whether infection requires pretreatment with medroxyprogesterone, 6-week-old female cotton rats were treated with 4-mg/kg medroxyprogesterone or sham injected and were infected intravaginally 24 h later with a cotton swab containing 10^2, 10^3, or 10^4 PFU of HSV-2(G) for 30 min. Animals were observed daily for 3 weeks and twice weekly for an additional 10 weeks. Almost all of the animals (29/30) developed lesions by day 12, independent of whether they received medroxyprogesterone 24 h prior to infection (Fig. 1). Epithelial lesions developed at the vaginal opening beginning 4 days after infection and increased in size to a maximum at day 14 (Fig. 2). The histological appearance of the lesions was characterized by ballooning degeneration of squamous epithelium, polymorphonuclear infiltration of damaged epithelium, and florid polymorphonuclear vaginitis, similar to the appearance of HSV-2 lesions in humans (data not shown). The size and number of the primary lesions increased (data not shown) with larger amounts of inoculum, while the duration of lesions did not vary (Fig. 2). Additionally, the extent of inflammation surrounding the vaginal wall appeared to increase with larger amounts of inoculum (data not shown). None of the animals developed significant neurological findings (e.g., hind-limb paralysis) requiring sacrifice, and no animals died during the 21 days of initial observation.

Cotton rats undergo spontaneous and induced clinical reactivation of genital herpes. The genital tract lesions resolved in all of the female rats by day 26 and subsequently 60 to 70% of the animals, independent of initial inoculum dose, developed spontaneous clinical recurrences with lesion formation. The lesions associated with reactivation tended to be smaller than those observed during primary infection (Fig. 3). The time course of spontaneous reactivation is illustrated for 15 rats with spontaneous recurrences (Fig. 4). To determine if reactivations could also be experimentally induced, 19 animals received a single intramuscular dose of dexamethasone (4 mg/kg) on day 105 postinfection and were observed for an additional 21 days. Recurrent lesions were seen at a rate that was two- to threefold higher than the rate of spontaneous reactivation (Fig. 5).

Primary HSV infection is associated with dissemination to extragenital tissues. Rather than inserting a cotton swab into the vagina for 30 min, we also examined the susceptibility to HSV infection if cotton rats were inoculated intravaginally with 30 μl of virus suspension with a pipette, as this is the protocol typically used in murine studies. No animals were pretreated with medroxyprogesterone. The animals were observed for 2 months to document the incidence of primary and recurrent lesions. Using this mode of viral inoculation, the dose required to achieve comparable rates of primary clinically apparent infection was higher, with 90% of animals receiving 10^5 PFU, 40% receiving 10^4 PFU, and 70% receiving 10^3 PFU developing primary lesions (Fig. 6). Spontaneous recurrences of lesions were also observed independent of whether the initial inoculum was delivered using a pipette or a cotton swab.

In humans, primary genital herpes infections may be associated with constitutional symptoms, aseptic meningitis, and clinically asymptomatic spread of the virus to liver, lungs, and kidneys. To determine whether primary HSV-2 infection is associated with evidence of viral spread in the cotton rat, several animals from each group were sacrificed at different days postexposure, and organs were harvested for histopathology and for viral detection by PCR. Figure 7A depicts results obtained from one animal 3 days post-vaginal inoculation with 10^5 PFU of virus. Viral DNA was detected in vaginal swab, kidney, brain, lumbosacral cord (including dorsal root ganglia),

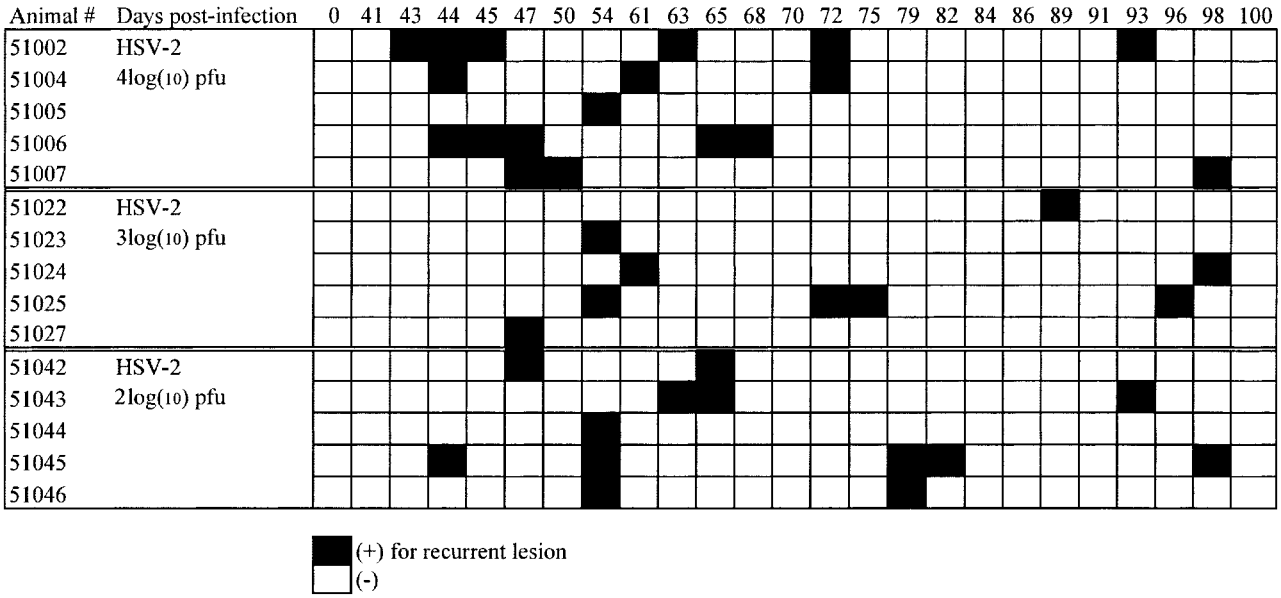

FIG. 4. Time course of spontaneous clinical recurrences of genital lesions in each cotton rat.

and liver but not in sciatic nerve or lung. Table 1 shows cumulative results obtained from several animals representing each group at different times p.i. Viral DNA was readily detected in vaginal swabs on days 3, 5, and 7 p.i. and could also be detected in the liver, lung, kidney, lumbosacral cord, and brain on the same days; viral DNA was only detected in the lung on day 3 but not at the later times. Additionally, within the same experiment, brain and lumbosacral cord were examined 100 days postinfection in one animal; viral DNA was detected in lumbosacral cord but not the brain, suggesting a state of latency in cotton rat dorsal root ganglia (Fig. 7B). The presence of comparable amounts of DNA was confirmed by GAPDH amplification (data not shown).

Different assays are used both in clinical studies and in animal models to detect the presence of HSV in the genital tract and include the presence of clinical lesions, detection of infectious virus by culture, or presence of viral DNA by PCR. To examine the time course of viral detection by PCR, culture, and the presence of clinical symptoms, we followed one cotton rat clinically and obtained daily vaginal swabs. Results indicated that virus was detected in the vagina by either PCR or culture of vaginal swabs prior to the onset of clinical lesions (Fig. 8). In addition, intermittent spontaneous viral shedding was observed in the absence of clinical lesions. Moreover, virus was also detected by PCR and culture prior to the onset of a clinical recurrence; these findings are consistent with those observed with human HSV infection.

Efficacy of topical microbicides against HSV-2 infection in the cotton rat model. Several candidate microbicides are currently in large phase IIb/III clinical trials. Two of these trials

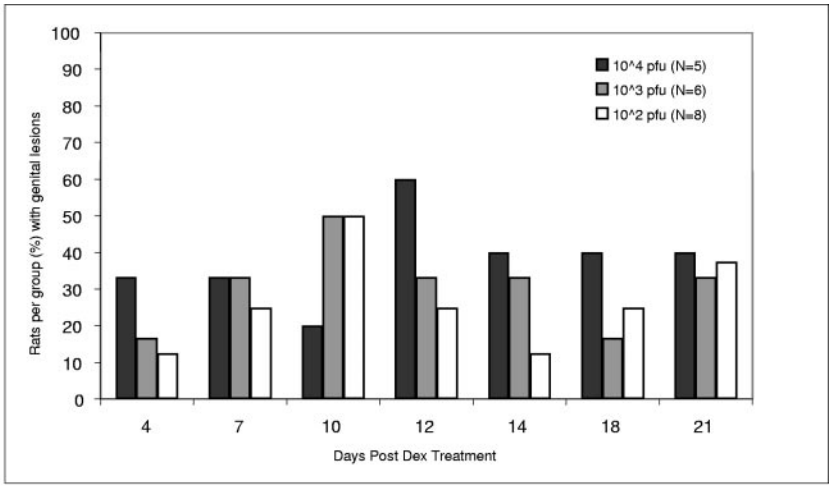

FIG. 5. HSV recurrences can be induced following dexamethasone (Dex) treatment. The percentage of cotton rats that developed clinical recurrences following treatment intramuscularly with dexamethasone (4 mg/kg) administered 105 days p.i.

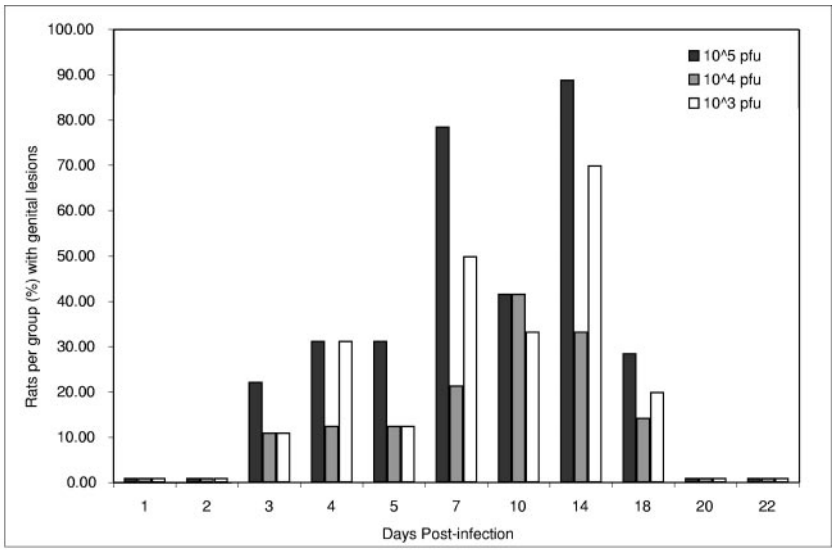

FIG. 6. Primary genital herpes following intravaginal application of virus introduced by pipette. Percentages of cotton rats developing clinical lesions at the indicated times (in days) following intravaginal inoculation with HSV-2(G) introduced by pipette (eight rats per group) are shown.

involve the candidate drug PRO 2000, a synthetic naphthalene sulfonic acid polymer, that interacts with viral glycoproteins HIV gp120 and HSV-2 glycoprotein B to prevent viral entry and infection (6). PRO 2000 gel has been shown to inhibit vaginal simian/human immunodeficiency virus infection in macaques (36), HSV-2 infection in mice (3), and gonorrhea in mice (30). To examine its effect in the cotton rat, cotton rats were pretreated with PRO 2000 (4% gel), a matched placebo gel, or PBS; 15 min later, the rats were challenged by intravaginal inoculation with 10^4 PFU of HSV-2 G (100-μl suspension). All of the cotton rats that received PRO 2000 gel (10 rats) were protected from infection and disease, as shown by the absence of any detectable virus by determining titers by plaque assay or detecting DNA by PCR of vaginal swabs and the absence of clinical signs of disease (Table 2). In contrast, 8/10 rats that received vehicle and 9/10 rats that received PBS developed primary lesions (Table 2). The animals were observed daily for 14 days and three times a week for additional 46 days. Representative PCR results obtained from three animals from each group (PRO 2000, vehicle, and PBS) demonstrate that no virus was detected from the PRO 2000 group by nested PCR, whereas virus was readily detected from vaginal swabs in the vehicle- or PBS-treated group (Fig. 9).

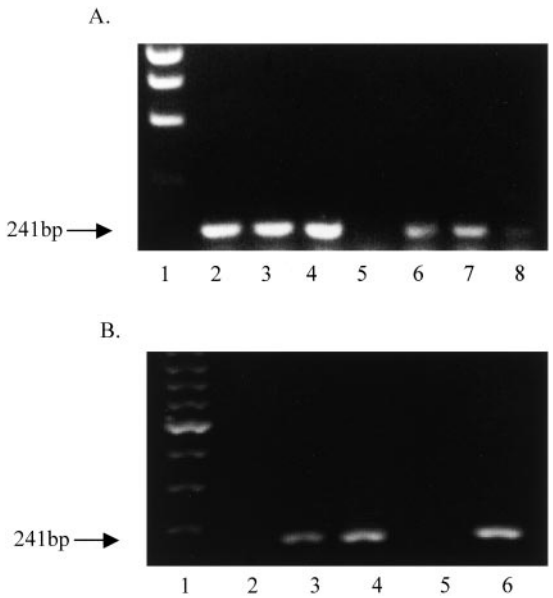

FIG. 7. Tissues were extracted and examined by PCR for HSV DNA. (A) Results from one representative animal that was sacrificed 3 days p.i. (10^5 PFU). Lane 1, DNA ladder; lane 2, vagina; lane 3, kidney; lane 4, brain; lane 5, sciatic nerve; lane 6, lumbosacral cord; lane 7, liver; and lane 8, lung. (B) Brain and lumbosacral cord (including dorsal root ganglia) were extracted from an animal 100 days p.i. with 10^5 PFU HSV-2. Lane 1, DNA ladder; lane 2, negative control; lane 3, HSV DNA (positive control); lane 4, HSV DNA (positive control); lane 5, brain; lane 6, lumbosacral cord.

DISCUSSION

These studies indicate that the cotton rat provides a potentially biologically superior model of human genital disease relative to the mouse or guinea pig and may prove an excellent model to test candidate microbicides for efficacy and safety. The salient features of this model are that vaginal infection does not require hormonal manipulation, primary infection is associated with spread of virus to other organs known to be targets of HSV dissemination in humans such as the liver, animals recover from primary infection but are prone to spontaneous clinical recurrences that mimic human disease, and infection is prevented by pretreatment with a candidate topical microbicide. Additional advantages include the availability of reagents to study the mucosal response to HSV and to microbicides, the ability to induce reactivation experimentally with dexamethasone, the potential to study vertical and horizontal transmission, and the possibility of developing the cotton rat as a model of coinfection with other STI.

Primary infection in cotton rats closely mimicked that ob-

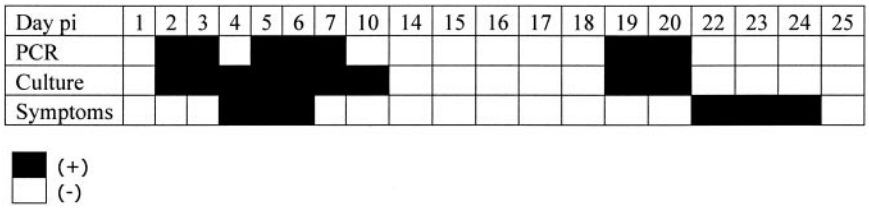

FIG. 8. Representation of one cotton rat infected with 10^4 PFU of HSV-2(G), monitored for HSV by PCR culture of vaginal washes and by clinical symptoms.

served in humans. The pattern of dissemination is similar to that observed in human disease, in which extragenital complications of primary HSV-2 infection include aseptic meningitis and dissemination to liver, lung, and kidney. Dissemination to these other sites is usually clinically silent but may cause disease in immunocompromised hosts, such as neonates, transplantation recipients, and persons with AIDS.

Although both mouse and guinea pig models have yielded important information, neither of these models fully recapitulates human disease. This is particularly problematic with the mouse, where the model is only consistently permissive for HSV following treatment with medroxyprogesterone. It had been presumed that the major impact of hormonal treatment is to induce thinning of the vaginal epithelium, rendering the mouse more susceptible to pathogens. However, this notion has been recently challenged. Kaushic and colleagues demonstrated that medroxyprogesterone treatment of mice resulted in a 100-fold increase in susceptibility to genital HSV-2 compared to untreated mice at diestrous phase (12). Furthermore, long-term effects of medroxyprogesterone treatment included reduction in protective immunity to HSV-2. Moreover, the expression of nectin-1, a key coreceptor for HSV entry, is expressed on the superficial epithelial cells of the mouse vagina only during the susceptible diestrous and proestrous phases of the estrous cycle. In contrast, human vaginal epithelium expresses nectin-1 at all stages of the menstrual cycle (17). These findings again highlight differences between a mouse model and human disease.

The morbidity and mortality associated with HSV infection in the murine model preclude its use to study horizontal or vertical transmission or to evaluate strategies designed to prevent reactivation. Preventing reactivation has been increasingly recognized as an important public health priority. Epidemiological studies consistently demonstrate that recurrent HSV increases the risk of HIV acquisition, enhances HIV replication, and itself can be associated with serious morbidity and mortality, especially among neonates and immunocompromised hosts (5, 20). Longitudinal studies indicate that clinical and subclinical viral reactivation occurs relatively frequently and is associated with sexual transmission of HSV, even between monogamous individuals. Although prophylactic antiviral therapy reduces the frequency and degree of viral shedding and lowers the transmission rate in discordant monogamous couples, transmission still occurs (8). With respect to HIV, a study conducted among a cohort of monogamous HIV-1 discordant couples from Rakai, Uganda, demonstrated that the per-contact risk of HIV acquisition, which averaged 0.0011, was fivefold higher if the susceptible partner was HSV-2 seropositive rather than seronegative (9). Although the probability of HIV acquisition was highest if the susceptible partner had genital ulcer disease, the risk was also increased with asymptomatic HSV, suggesting that subclinical reactivation is almost as important as clinical disease in increasing the risk of HIV acquisition. In contrast, symptoms of urethritis and laboratory-confirmed diagnoses of gonorrhea, *Chlamydia* infection, and trichomoniasis did not increase HIV risk. Additionally, several studies have shown that subclinical HSV reactivation is associated with increased replication of HIV. The quantity of HSV DNA correlates with HIV-1 RNA in cervicovaginal secretions of women without genital lesions (19, 20). Taken together, these epidemiologic studies support the hypothesis that control of HSV-2 reactivation may help reduce HIV transmission.

The cotton rat provides an animal model to test strategies designed to reduce reactivation. Specifically, we found that spontaneous clinical recurrences occur as frequently as every 9 days, are self limited with smaller lesion size, and are often preceded by virus being detected in vaginal swabs by PCR or culture. Virus was not always detected at the time that genital lesions appeared. Similar results were reported in a study correlating symptoms of recurrences and viral shedding (35). Moreover, reactivation is induced in >50% of cotton rats fol-

TABLE 1. Number of animals in which HSV was detected by PCR of tissue harvested 3, 5, or 7 days following viral infection with the indicated viral inoculum[a]

Tissue	Viral inoculum at indicated day p.i.											
	10^5 PFU			10^4 PFU			10^3 PFU			Total		
No. of days p.i.	3	5	7	3	5	7	3	5	7	3	5	7
Vagina	7/7	1/1	1/2	5/5	ND	1/1	ND	1/1	2/2	12/12	2/2	4/5
Liver	2/2	2/2	1/1	1/2	2/2	2/2	2/2	2/3	1/1	5/6	6/7	4/4
Lung	2/2	0/2	0/2	1/2	0/2	0/1	1/2	0/2	0/1	4/6	0/6	0/4
Kidney	1/2	0/2	1/2	ND	2/2	2/2	1/2	1/1	1/2	2/4	3/5	4/6
LS cord	2/2	0/2	1/1	2/2	0/2	0/1	1/2	0/2	0/1	5/6	0/6	1/3
Brain	2/2	1/2	1/2	ND	1/2	ND	0/2	0/1	1/1	2/4	2/5	2/3

[a] ND, not done; LS, lumbosacral. Values for vagina, liver, and other tissues are the number of samples testing positive for HSV/total number of samples tested.

TABLE 2. PRO 2000 protects cotton rats from infection and disease[a]

Group	Virus isolated by culture:	Viral DNA detected (PCR)	No. of genital lesions
PBS	10/10	10/10	9/10
Vehicle	10/10	10/10	8/10
4% PRO 2000	0/10	0/10	0/10

[a] Values indicate number of rats testing positive/total number tested.

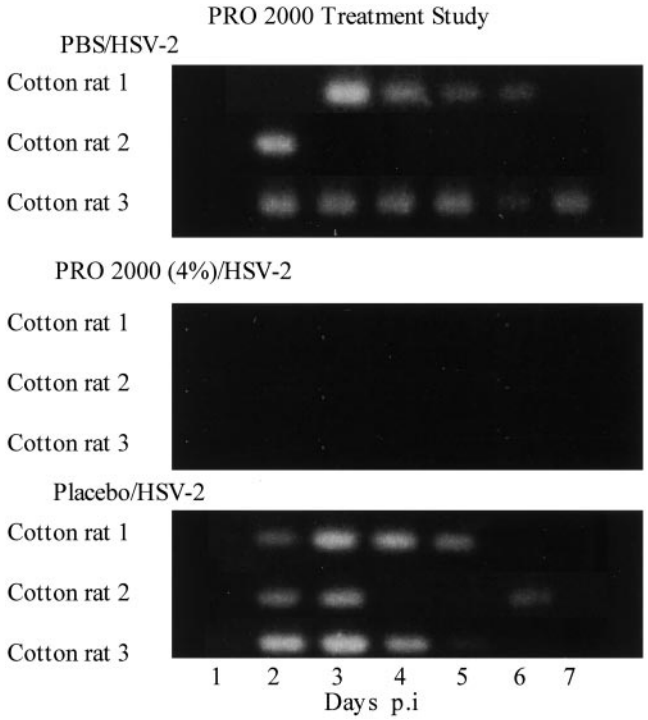

FIG. 9. Cotton rats (10 per group) were treated with 4% PRO 2000, matched placebo gel, or PBS and then challenged 15 min later with 10^4 PFU of HSV-2(G) introduced by pipette. Vaginal swabs were collected for 7 days for detection of HSV by PCR; results for vaginal swabs from three animals per group are shown.

lowing dexamethasone therapy, which may provide a model for reactivation in the setting of immune suppression. Although spontaneous recurrences also occur in guinea pigs, the number of reagents available for studying mucosal immunity in guinea pigs is limited. In contrast, there has been recent expansion and development of a library of cotton rat genes that have been sequenced, including genes expressing cytokines, chemokines, cell surface markers, transcriptional factors, and membrane-associated proteins. This has fostered the development of a wide array of reagents, which include primers and probes, recombinant proteins, polyclonal and monoclonal antibodies, and enzyme-linked immunosorbent assays for cytokines, chemokines, and other important cell surface molecules and proteins. Recognition that repeated microbicide exposure may alter the genital tract environment, leading to increased susceptibility to HIV, as was observed for nonoxynol-9 (33), has prompted investigators to prioritize a rigorous evaluation of the mucosal responses to candidate microbicides as a priority in their preclinical evaluations (13). However, animal models that provide surrogate markers of safety that correlate with clinical outcomes have not been identified. These studies suggest that the cotton rat may provide an appropriate model for evaluation of the safety and effectiveness of candidate microbicides and other preventive strategies.

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