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Intravaginal administration of herpes simplex virus type 2 to mice leads to infection of several neural and extraneural sites

Margaret B Parr and Earl L Parr

Department of Anatomy, School of Medicine, Southern Illinois University, Carbondale, Illinois, USA

Female mice have been used extensively to study mucosal immunity against herpes simplex virus type 2 (HSV-2) infection of the vagina, but comparatively little is known about the spread of this virus to other tissues. Here the authors have used immunolabeling to demonstrate that HSV-2 infected the vaginal epithelium; the epithelium covering the vulva, perineum, and anal canal; and perineal hair follicles and sebaceous glands. The kinetics and basal localization of the immunolabeling indicated that the virus spread horizontally within the epithelial layer, starting in the vagina and then proceeding to the distal epithelial sites. HSV-2 also spread from the vagina to multiple neuronal sites including the paracervical ganglia (PCG), which are the major autonomic ganglia of the pelvis. The authors demonstrated both sympathetic and parasympathetic neurons in the PCG by labeling of acetylcholinesterase and tryosine hydroxlyase, and noted that infection was limited mainly or entirely to parasympathetic neurons. Infection of the PCG was correlated with the presence of virus in the autonomic ganglia in the walls of the rectum and urinary bladder, which in turn correlated with distention of these organs and retention of urine and feces. HSV-2 infection was also detected in cell bodies and axons in the lumbosacral sympathetic chain, in lumbosacral dorsal root ganglia, and in the dorsal portions of the lumbar spinal cord. Collectively, the data show that vaginal HSV-2 infection in mice leads to subsequent infection of multiple neural and epithelial sites. This information should be useful for development of a mouse model that can be used to study HSV-2 latency and for development of therapeutic vaccines to prevent recurrent infections. *Journal of NeuroVirology* (2003) **9,** 594–602.

Keywords: herpes simplex virus type 2; mouse; pathogenesis; vagina

Introduction

Herpes simplex virus (HSV)-2 is a sexually transmitted pathogen that infects the genital tract and contributes significantly to morbidity in humans, with infections being particularly severe in neonates and immunocompromised individuals (Quinnan *et al*, 1984; Greenblatt *et al*, 1988; Whitley *et al*, 1991). The prevalence of HSV-2 infections is increasing worldwide and over 20% of the adult US population is infected with the virus (Corey and Spear, 1986a, 1986b; Nahmias *et al*, 1990). In women, HSV-2 infects the mucosa of the lower part of the genital tract, sensory neurons, and lumbosacral dorsal root ganglia where it becomes latent and persists for the life of the host. Reactivation of latent virus, which may be triggered by exposure to ultraviolet (UV) light, fever, tissue and/or nerve damage, or immunosuppression, is followed by axonal spread of the virus back to the site of infection, with the formation of lesions in the genital tract and adjacent tissues (Corey and Spear, 1986a, 1986b).

A vaccine to prevent HSV-2 infections in the genital tract and subsequent latency would greatly contribute to preventive health care but is problematic because it requires sterile immunity. However, development of a therapeutic vaccine that could suppress

Address correspondence to Margaret B. Parr, Department of Anatomy, School of Medicine, Southern Illinois University, Carbondale, IL 62901-6523, USA. E-mail: mparr@siumed.edu

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reactivation of latent virus may be more feasible and deserves thorough investigation. Currently, the only animal model for studies of recurrent herpetic disease in the genital tract uses the guinea pig (Stanberry, 1991). Female guinea pigs inoculated intravaginally with HSV-2 develop variable mucosal infection, establish latency in spinal ganglia, and subsequently develop recurrent disease (perineal lesions). However, this species is limited in its usefulness because of the paucity of inbred strains, limited availability of immunologic reagents, absence of genetically altered animals, cost, and variability in the initial infection. In addition, 20% to 80% of infected guinea pigs die from the initial infection (Lukas *et al*, 1975; Scriba, 1976; Stanberry *et al*, 1982). Importantly, the guinea pig model may have disadvantages for immunologic studies. DaCosta *et al* (2001) have pointed out that subunit vaccines composed of HSV-2 glycoproteins protected guinea pigs against primary and recurrent genital disease but were ineffective or only marginally effective in human clinical trials. These investigators suggested that the mouse might provide a more suitable model for immunologic studies.

Mice inoculated intravaginally with HSV-2 develop infection in the vaginal epithelium and probably other tissues that leads to death of most of the animals within 10 days. This lethal challenge model has been useful for studies of host defenses and to evaluate experimental HSV vaccines (Parr and Parr, 1997, 1999), but it could not be used to explore HSV latency and recurrent infection. Substantial information about HSV latency and recurrence has been obtained using nonlethal HSV-1 infection of the mouse ear (Hill *et al*, 1980), eye (Cantin *et al*, 1999), and skin (Simmons and Nash, 1984), but the relevance of these studies to HSV-2 infection in the genital tract is unknown.

Some attempts have been made previously to establish a mouse model for recurrent HSV-2 genital disease. Wroz and Rapp (1985) used 4- to 6-week-old C57BL/6 mice, a strain thought to be relatively resistant to HSV infection (Zawatzky *et al*, 1981). The mice were infected intravaginally with a high viral dose followed by immunosuppression, but few mice survived. Martin and Reed (1986) used 3- to 12-week-old outbred or BALB/c mice inoculated intravaginally with a lower viral dose of a less virulent strain of HSV-2. Some reactivation of latent HSV-2 was detected in the genital tract and central nervous system of surviving mice, but variability in the primary infection limited the usefulness of this model.

One of the main factors that frustrated early attempts to develop an effective mouse model for recurrent herpetic disease was variability in the number of mice that became infected after intravaginal inoculation of HSV-2. This obstacle can be overcome by administration of a synthetic progestin (Depo-Provera, DP) prior to inoculation of virus (Parr *et al*, 1994). This method provides essentially 100% infection and was based on earlier reports that pregnant mice were much more susceptible to vaginal HSV-2 infection than nonpregnant adult mice (Overall *et al*, 1975; Baker and Plotkin, 1978). Histologically, the vagina of DP-treated mice is lined by mucus-secreting columnar epithelium overlying two to three layers of proliferative cells. Although this is morphologically unlike the human vagina, it closely resembles the endocervical epithelium, which is thought to be a major site of HSV-2 infection in women (Parr *et al*, 1994; Cooper, 2000).

A second obstacle to the use of mice for studies of recurrent genital herpes infection is that mice do not generally survive the infection. However, recent experiments in our laboratory have shown that HSV-2 introduced ivag into DP-treated adult mice followed 3 days later by treatment with valacyclovir plus passive administration of monoclonal antibody against HSV glycoprotein D resulted in 27/29 mice becoming infected, 26/27 surviving to 2 weeks, and 21/27 surviving to 8 weeks after infection. The 3-day interval between infection and antiviral therapy may be sufficient time for the virus to reach neural tissues and establish latent infections. The ability to infect mice uniformly with intravaginally administered HSV-2 and to rescue a majority of them from death may permit development of a mouse model for studies of genital HSV-2 latency, recurrence, and therapeutic vaccines. Such a model could provide information what would help us to assemble a more comprehensive and coherent picture of genital herpes infections.

In developing this model, it is important to understand how long it takes virus to infect dorsal root ganglia after intravaginal inoculation and to determine other possible sites of infection. Here we show that after intravaginal administration, HSV-2 initially infects the vaginal epithelium and then spreads to the epithelium of the vulva, anus, and perineum; the epithelium of hair follicles and sebaceous glands in the perineum; paracervical ganglia; autonomic ganglia in the walls of the urinary bladder and rectum; sympathetic chain ganglia; lumbosacral dorsal root ganglia; and the spinal cord.

Results

Neurological disease

Intravaginal inoculation of HSV-2 into DP-treated mice resulted in clinically evident infection in essentially all mice. The animals appeared normal up to days 5 to 6 post infection (p.i.), at which time they showed ruffled fur, swollen red vulva, urine and fecal retention, perineal skin lesions, and hair loss. By days 7 to 8 p.i., the mice became lethargic, had feeble movements, and often had hunched backs. By days 8 to 9 p.i., mice developed hindlimb paralysis, at which time they were sacrificed.

Table 1 Virus infection after intravaginal inoculation of HSV-2

Sites of infection	Days after inoculation of virus						
	2	3	4	5	6	7	9
Vagina	**8/8***	**4/4**	**7/7**	**4/4**	**2/4**	**1/4**	**1/4**
Vulva	**2/6**	**1/3**	**5/5**	**1/1**	**1/3**	**1/4**	**1/2**
Dorsal root ganglia	**2/6**	**1/4**	**3/8**	**3/4**	**3/4**	**8/8**	**4/4**
Paracervical ganglia	0/4	0/4	**3/4**	**2/2**	**4/4**	**6/6**	**0/4**
Urethra	0/8	0/3	**1/8**	**3/4**	**0/4**	**2/4**	**0/5**
Urinary bladder	0/4	0/4	**3/3**	**4/4**	**4/4**	**4/4**	**NA**
Rectum	0/8	0/4	**3/8**	**4/4**	**4/4**	**8/8**	**1/4**
Perineum	0/6	0/4	**2/6**	**3/3**	**0/3**	**7/8**	**0/4**
Hairs in genital skin	0/6	0/4	0/6	**3/3**	**1/4**	**7/8**	**0/4**
Spinal cord	0/5	0/4	0/8	**3/4**	**3/4**	**3/3**	**1/1**
Sympathetic chain	NA	NA	NA	NA	**5/5**	NA	NA

*Proportion of mice showing immunolabeling of HSV-2. NA, not available.

Vagina

Two to 5 days after vaginal inoculation with HSV-2, virus-infected cells were observed in the vaginal epithelium by immunostaining in all animals tested (23/23; Table 1). The infection occurred in patches that involved the full thickness of the shallow stratified epithelial layer; the underlying stroma was generally uninfected, although in a few cases punctate labeling, possibly in neurons, was observed in the connective tissue a short distance below the epithelial basement membrane. Areas of infected epithelium were sloughed off into the vaginal lumen as early as day 2 p.i. HSV-2 immunolabeling of the vaginal epithelium was evident from the vaginal fornix to the distal region of the vagina at the introitus. No HSV-2 labeling was detected in the cervix or uterine horns in 21 mice examined from days 2 to 9 p.i. By day 4 p.i., HSV-2 labeling was seen in nerves in the adventitia outside the vagina. By day 6 p.i., the infection in the vagina appeared to be resolving, with less HSV-2 labeling in the vaginal epithelium and no signs of sloughing of infected epithelium. The apparent resolution of vaginal infection and repair of the epithelium was accompanied by a marked influx into the vagina of CD45+ cells that had the morphological appearance of lymphocytes.

Vulva

The stratified columnar epithelium that is characteristic of the DP-treated vagina changed abruptly to a thick cornified stratified squamous epithelium that lined the vulva at the introitus. Unlike the vaginal epithelium that was infected in all mice up to day 6 p.i., the vulvar epithelium showed HSV-2 labeling in only a portion of the mice between days 2 and 9 p.i., with greatest incidence of labeling on day 4. As in the vaginal epithelium, patches of vulvar epithelium were infected, but initially the virus staining did not involve the full thickness of this epithelium. Instead, only the basal portion of this epithelium was infected and most often just at the junction with the infected vaginal epithelium. This pattern of immunolabeling suggested that virus from the infected vaginal epithelium spread to the vulvar epithelium via the basal cells, leaving the luminal cells generally free of infection. By day 4 p.i., areas of infected vulvar epithelium sloughed off, leaving denuded regions, and HSV-2 continued to be observed in numerous nerves throughout the lamina propria; an influx of CD45+ cells was also evident at this time.

Perineum, hairs, and sebaceous glands

The vulva continues onto the surface of the body as the perineum that extends about 0.5 cm and is located between the vagina and anus (Eckstein and Zuckerman, 1956). The perineal epithelium consists of a thin, cornified, stratified epithelium interspersed with hairs and sebaceous glands. Basal regions of the perineal epithelium became infected by day 4 p.i. The pattern of HSV-2 immunolabeling suggested that virus in the infected basal cells of the vulvar epithelium spread to the basal cells of the perineal epidermis. By day 7 p.i., regions of the perineal epidermis were sloughed off, resulting in skin lesions that showed variable signs of repair. Hairs and sebaceous glands in the perineal epithelium showed HSV-2 immunolabeling on days 5 to 7 p.i. (Figure 1**A**). The pattern of staining suggested that virus in infected basal perineal epithelial cells spread to the inner and outer hair sheaths and sebaceous glands. Infection of the hairs resulted in significant hair loss in the perineum. Viral infection of the perineal epidermis, hairs, and sebaceous glands was accompanied by a modest influx of CD45+ cells.

Paracervical ganglia

The paracervical ganglia (PCG, pelvic ganglia) are autonomic ganglia located outside the vaginal fornix and uterine cervix. They showed HSV-2 labeling in cell bodies and adjacent axons between days 4 and 7 p.i. in 15/16 mice examined (Figure 1**B**). Infection of the ganglia was accompanied by an influx of CD45+ immune cells. Using thyrosine hydroxylase (TH) as a marker for adrenergic neurons and choline acetyltransferase (Chat) as a marker for cholinergic neurons, we found labeling of cell bodies and axons in the PCG that were either Chat or TH positive, but predominantly Chat+. Double labeling of HSV-2 and Chat showed cell bodies that were either Chat+ and HSV-2+, or Chat+ and HSV-2−, (Figure 2). Double labeling of HSV-2 and TH showed cell bodies that were either TH+, HSV-2−, or TH−, HSV-2+. These observations suggest that many of the parasympathetic postganglionic neurons, but few, if any, of the sympathetic neurons of the PCG became infected with HSV-2 after intravaginal inoculation of the virus.

Urethra and urinary bladder

Coincident with viral infection of the PCG on day 4 p.i., HSV-2 labeling was observed in the autonomic ganglia and neurons in the wall of the urinary bladder

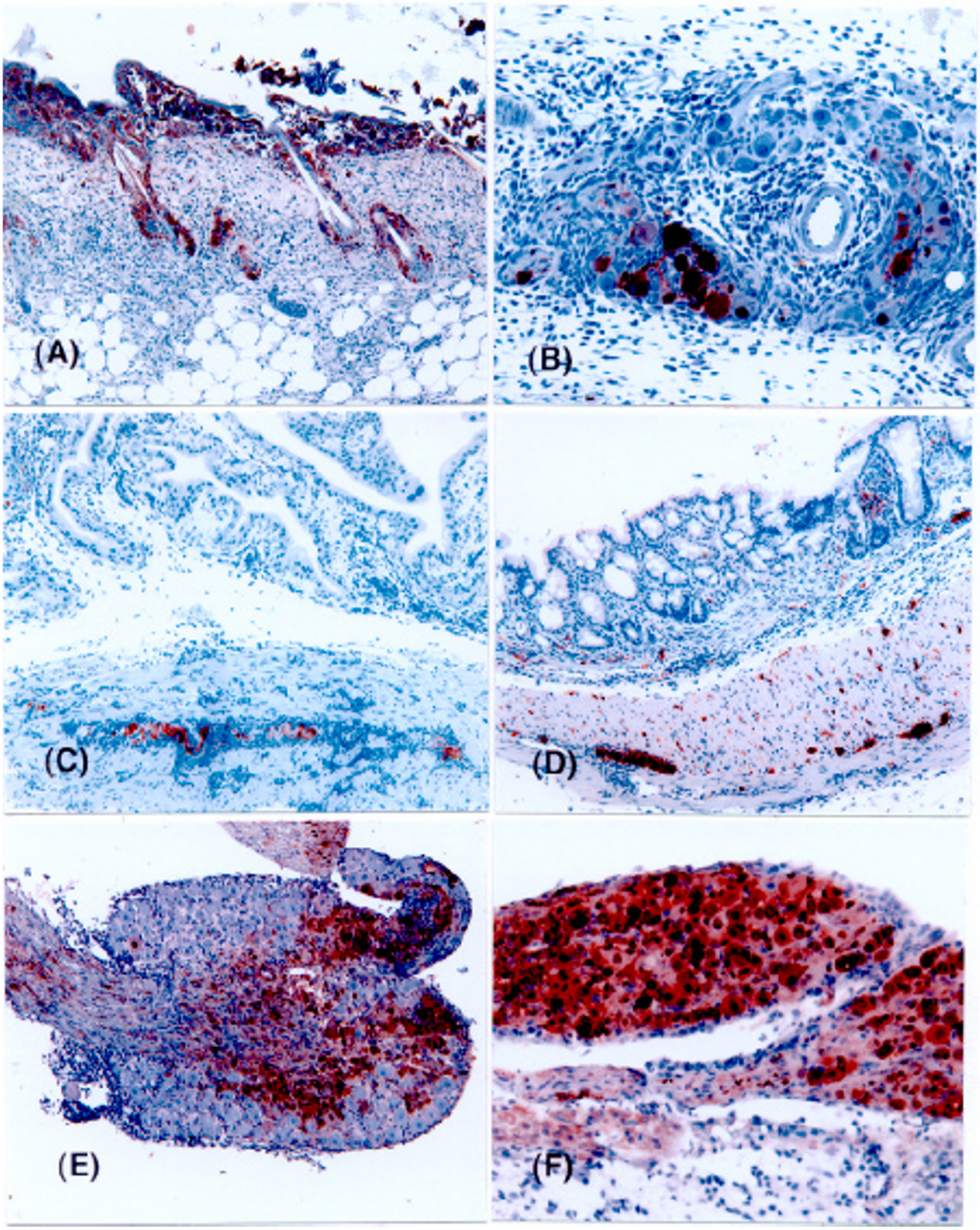

Figure 1 Immunolabeling of HSV-2 (brown reaction product) in various tissues 5 to 7 days after intravaginal inoculation. (**A**) Virus can be observed in the epithelium of perineal skin and inner and outer epithelial sheaths of hair follicles on day 7 p.i. (**B**) HSV labeling is present in cell bodies of the PCG located outside the cervix/vaginal fornix taken from a mouse on day 5 p.i. (**C**) HSV-2 labeling can be seen in nerves and autonomic ganglia in the wall of the urinary bladder. (**D**) HSV-2 labeling can be seen in Auerbach's plexus and nerves in the submucosa of the rectum. (**E**) Prominent HSV-2 was detected in spinal nerves and lumbar dorsal root ganglia. (**F**) Prominent HSV-2 was detected in nerves and ganglia in the sympathetic chain. **A**, **C**, **D**, and **E**, ×85; **B** and **F**, ×170.

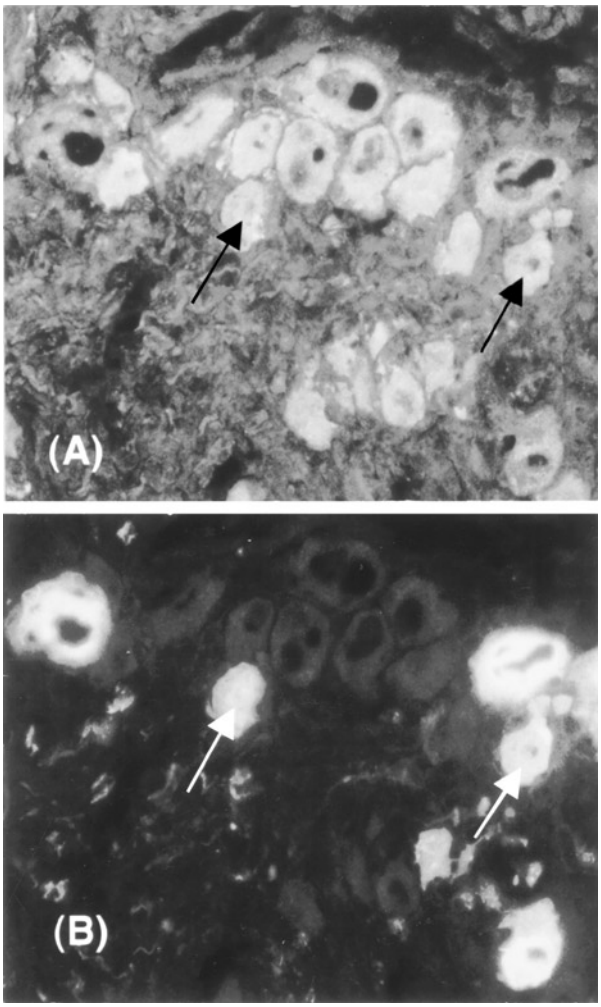

Figure 2 Double-immunofluorescent labeling of Chat (**A**) and HSV-2 (**B**) in cell bodies in the PCG from a mouse on day 6 p.i. Cell bodies labeled for HSV-2 colabeled with those labeled for Chat (*arrows*), whereas many that were Chat+ were HSV-2−. ×250.

in 15/15 mice between days 4 and 7 p.i. (Figure 1**C**). Infection was accompanied by an influx of CD45+ cells. Some of these mice showed traces of infection in the epithelium lining the distal urethra and urinary bladder.

Rectum and anal epithelium
Infection was observed in Auerbach's and Meissner's plexuses in the wall of the rectum from days 4 to 9 p.i. in 20/28 mice examined. Only occasional labeling was present in what appeared to be neurons in the lamina propria, and no labeling was seen in the columnar epithelium lining the rectum. This epithelium changes abruptly into stratified squamous epithelium in the anal canal; in some cases, the stratified epithelium at this site was HSV-2+ but the adjacent columnar epithelium remained free of virus. Infection in the rectum was accompanied by an influx of CD45+ cells. No HSV-2 labeling was seen in the ascending colon in 21 mice examined from days 2 to 9 p.i., and the number of CD45+ cells in this part of the gastrointestinal tract was similar to that seen in non-infected control mice.

Dorsal root ganglia
Dorsal root ganglia and adjacent nerves from the lumbar and sacral regions of the spinal cord showed infection with HSV-2 throughout the study period. On days 2 and 3 p.i., only one to two cell bodies from one to two ganglia were infected, but this increased markedly from day 4 onwards (Figure 1**E**). Few, if any, CD45+ cells were observed in the ganglia.

Spinal cord
The white and gray matter in the posterior portions of the lumbar spinal cord were HSV-2+ by days 5 to 9 p.i., but all portions of the sacral cord examined were negative. Very few, if any, positively labeled neurons were detected in the ventral part of the spinal cord. Spinal cord regions were identified by reference to an atlas (Sidman *et al*, 1971). Occasionally, ependymal cells lining the central canal of the spinal cord were infected. Few, if any, CD45+ cells were observed in the spinal cords.

Sympathetic chain
HSV-2 immunolabeling was detected in many cell bodies and axons of lumbosacral sympathetic chain ganglia in 5/5 mice on day 6 p.i. (Figure 1**F**). No labeling of HSV-2 was observed in the adrenal medulla in 21 mice examined from days 2 to 9 p.i. Immunolabeling of CD45 was not carried out on the sympathetic chain because of the limited supply of tissues. The presence of virus in the postganglionic neurons of the sympathetic chain ganglia contrasts with the absence of virus in the postganglionic sympathetic neurons of the PCG. Neurons in the sympathetic chain ganglia evidently became infected by spread of virus from perineal epidermis, hair follicles, and sebaceous glands to the adjacent endings of the sympathetic neurons, then by retrograde transport to the lumbosacral cell bodies.

Discussion

This study documents an extensive spread of HSV-2 infection to multiple neural and extraneural sites after intravaginal inoculation in mice (Figure 3). Viral infections were noted in the epithelia covering the vulva, perineum, and anus, as well as in perineal hair follicles and sebaceous glands. The localization and kinetics of this HSV-2 immunolabeling suggested that virus spread horizontally within the epithelial layers, starting from the vagina and then proceeded distally along the base of the epithelium to the vulva and perineum, and from there to the associated hairs and sebaceous glands. This infection was associated with significant hair loss and skin lesions. Although it is possible that the vulvar and perineal

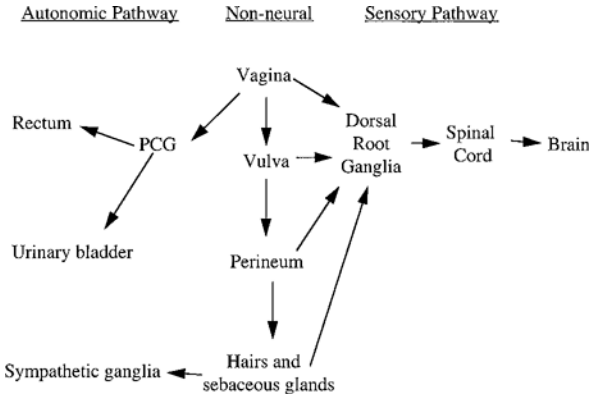

Figure 3 Possible pathways of HSV-2 after intravaginal challenge.

epithelia became infected by exogenous free virus derived from the infected vagina, this seems unlikely because intact keratinized squamous epithelium has been shown to resist HSV-2 infection (Parr *et al*, 1994) and direct application of virus onto the genital skin of guinea pigs did not cause infection (Stanberry *et al*, 1982). Stanberry *et al* (1982) have suggested that genital skin lesions observed in guinea pigs after intravaginal inoculation of HSV-2 might be due to dissemination of virus via sensory neurons. However, we have observed genital skin lesions and hair loss in approximately 15% of mice that were inoculated intravaginally with thymidine kinase–deficient HSV-2, which does not replicate in nerve cells (Kosz-Vnenchak *et al*, 1990; Ho, 1992; Jacobson *et al*, 1993; Stroop *et al*, 1994). These lesions continued to expand for several weeks after virus had been cleared from the vagina (unpublished data). Thus, several routes of viral dissemination may be involved in the vulvar and perineal skin lesions and hair loss that occurs after vaginal HSV-2 infection.

The present study also showed that HSV-2 spread from the vagina to multiple neuronal sites including the PCG (pelvic ganglia, uterovaginal plexus), confirming and extending earlier reports (Walz *et al*, 1977; Sanjuan and Lascano, 1986; Fleck *et al*, 1993; Podlech *et al*, 1996). The PCG is the major autonomic ganglion of the pelvis, involving both the sympathetic and parasympathetic divisions of the nervous system (Hammarstrom, 1980). It receives inputs from the hypogastric nerve (mainly sympathetic) and the sacral splanchnic nerve (parasympathetic). Postganglionic neurons from the PCG have been shown to innervate the uterus, vagina, urinary bladder, and rectum (Costa and Furness, 1973; Mitchell *et al*, 1993). Although Sanjuan and Lascano (1986) have described the PCG in the mouse as sympathetic, we demonstrated both parasympathetic and sympathetic neurons in the PCG by labeling of Chat and TH, respectively, with a predominance of parasympathetic neurons. Virus infection in the PCG was limited mainly or entirely to parasympathetic neurons. Virus might have reached these neurons after uptake by postganglionic parasympathetic motor nerve endings in vaginovulvar tissues, although other routes of infection are possible.

The detection of HSV-2 in the PCG was correlated with the presence of virus in autonomic ganglia in the walls of the rectum and urinary bladder, and with distention of these organs and retention of urine and feces. It is well known that sacral parasympathetic neurons provide the major excitatory input to regulate pelvic visceral functions such as micturition and defecation. In guinea pigs, Stanberry *et al* (1982) reported that infected urinary bladders and transient urinary retention were common after vaginal inoculation of HSV-2. In humans, genital HSV infection has been reported to cause autonomic nervous system dysfunction in the pelvis, including urinary retention and constipation, as well as sensory deficits (Corey and Spear, 1986a, 1986b).

Our observations also indicate for the first time that HSV-2 inoculated into the vagina can lead to infection of cell bodies and nerve fibers in the lumbosacral sympathetic chain. One possible route of viral transmission to this site is by uptake and retrograde passage of virus in postganglionic sympathetic fibers that innervate the sebaceous glands, hairs, and associated blood vessels in infected perineal skin. This raises the interesting possibility that recurrent genital skin infections may emanate not only from dorsal root ganglia but also from lumbosacral sympathetic chain ganglia.

It is generally accepted that HSV-2 reaches dorsal root ganglia (DRG) via retrograde transport in sensory neurons that innervate the site of infection. In the present study, we observed HSV-2 in lumbosacral DRG and in the dorsal aspect of the lumbar spinal cord after vaginal infection in mice. Virus could have reached the DRG via sensory neurons innervating the vagina and passing through the PCG (Papka and McNeill, 1992), and/or via sensory neurons that innervate the vulva and perineum and reach the DRG through the pudendal nerves.

In spite of differences in the organization and functional roles of autonomic and sensory ganglia, it seems that both types may support a latent HSV-2 infection. There is convincing evidence that the DRG are a site of recurrent HSV-2 infection (Corey and Spear, 1986a), and Price (1977) has suggested that autonomic ganglia may serve as reservoirs for latent HSV in humans. In mice, the PCG may serve as a reservoir for latent HSV because these ganglia contain HSV-2 latency-associated transcripts (LATs) (Podlech *et al*, 1997) and infectious virus can be recovered from explanted PCG after several days in culture (Walz *et al*, 1977). It is not yet known whether HSV-2 can establish latency in sympathetic ganglia, or whether HSV-2 in the PCG can reactivate and travel down postganglionic neurons to be released at nerve terminals. The spread of infection to the nervous system in mice may be more extensive than in humans, but reports of urinary and fecal retention

in some infected women have been interpreted as HSV-2 infection of the autonomic nervous system (Corey and Spear, 1986a, 1986b). Thus, a full understanding of recurrent genital infection in women may require consideration not only of sensory neurons and dorsal root ganglia, but also of parasympathetic motor neurons via the PCG and possibly sympathetic motor neurons via the lumbosacral chain ganglia.

Materials and methods

Animals and virus

Eighty-five female BALB/c mice were purchased from Harlan/Sprague-Dawley, Indianapolis, Indiana, and used when they were approximately 10 to 12 weeks old. They were housed in compliance with all institutional and federal animal welfare requirements, and all experimental procedures were approved by the institutional Animal Care and Use Committee. Wild-type HSV-2 was generously provided by Dr. Mark McDermott, McMaster University, Hamilton, Canada (McDermott *et al*, 1984, 1987).

Experimental design

Mice were pretreated with 2.5 mg of DP (Upjohn, Kalamazoo, MI) in phosphate-buffered saline (PBS) subcutaneously in order to maximize viral infection (Parr *et al*, 1994). Six days later, the mice were anaesthetized with avertin and 20 μl of HSV-2 at 1.5×10^6 plaque-forming units (PFU)/ml was inoculated into the vagina using a micropipette. Mice were sacrificed at various times after intravaginal inoculation to collect tissues for immunolabeling, whereas other mice were monitored daily for signs of neurological disease as controls.

Tissues and immunolabeling

Tissues were removed from noninfected or infected mice between 2 and 9 days after intravaginal inoculation, fixed in 4% paraformaldehyde (4°C, overnight), processed, and embedded in paraffin. Histological sections were used for immunolabeling of HSV-2 or CD45. For HSV-2 labeling, sections were blocked in 2% fetal calf serum, incubated in rabbit anti–HSV-2 (1/1000, 60 min, 37°C; Dako, Carpinteria, CA), washed in PBS, treated with 0.5% hydrogen peroxide in methanol, washed in PBS, incubated in biotinylated goat anti–rabbit immunoglobulin G (IgG) (1/150, 20 min, room temperature, Vector Laboratories, Burlingame, CA), followed by streptavidin-peroxidase (Zymed Labs, San Franscisco, CA), and exposed to substrate (AEC kit; Zymed Labs). For CD45 labeling, sections were treated as above using rat anti-mouse CD45 (1/100, 60 min, 37°C, Pharmingen, San Diego, CA) and biotinylated rabbit anti-rat IgG (1/50, 20 min, room temperature, Vector Laboratories) as primary and secondary antibodies. Noninfected mice served as controls. Specificity of labeling was indicated by the absence of staining in control sections and when irrelevant rabbit IgG, normal serum, or biotinylated antibody were substituted for the primary antibodies. Sections were counterstained with Gill's haematoxylin and mounted in Gelmount (Biomeda, Foster City, CA).

The vaginal fornix/cervix were removed from four noninfected and eight infected mice (day 6 p.i.) and fixed with 2% paraformaldehyde (2 h, 4°C), embedded in O.C.T. (Tissue-Tek; Miles Scientific, Naperville, IL), frozen in isopentane cooled with liquid nitrogen, and stored at −70°C until needed. Cryostat sections (5 μm) were used for double immunofluorescent labelling of HSV-2 and either ChAT or TH. The sections were processed as follows: PBS, 2% fetal calf serum, goat anti-ChAT (1/100, 4°C, overnight, Chemicon International, Temecula, CA) or sheep anti-TH (1/200, 4°C, overnight, Novus Biologicals, Littleton, CO) in 2% fetal calf serum, PBS wash; fluorescein isothiocyanate (FITC)-conjugated rabbit anti–HSV-2 (1/20, 60 min, room temperature, Dako), and TRITC-conjugated donkey anti-goat IgG (1/150, 60 min, room temperature) or TRITC-conjugated rabbit anti-sheep IgG (1/150, 60 min, room temperature, Jackson ImmunoResearch Labs., West Grove, PA) in 2% fetal calf serum, PBS wash, and mounted in polyvinyl alcohol (Sigma, St. Louis, MO). Noninfected mice served as controls. Specificity of labeling was indicated by the absence of fluorescence in control sections and when normal rabbit IgG, goat IgG, or sheep IgG (Jackson Immuno Research Labs.) were substituted for the specific antibodies.

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